BIOSYNTHESIS, *IN VIVO* AND *IN VITRO* DEPOLYMERIZATIONS OF INTRACELLULAR MEDIUM CHAIN LENGTH POLY-3-HYDROXYALKANOATES

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ABSTRACT

Medium chain length poly-3-hydroxyalkanoates (mcl-PHA) is a biodegradable polymer made of different hydroxyalkanoates with (*R*)-configuration as monomeric units and a source of valuable biomaterials. Biosynthesis, *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA in *Pseudomonas putida* Bet001 grown on lauric acid was studied. Highest mcl-PHA fraction (> 50 % of total biomass) and cell concentration (8 g L^{-1}) were obtained at carbon-to-nitrogen (C/N) ratio 20, initial cell concentration 1 g L^{-1}, and 48 hours fermentation. The mcl-PHA comprised of 3-hydroxyhexanoate (C_6) (6.1 ± 2.0 mol%), 3-hydroxyoctanote (C_8) (40.1 ± 2.7 mol%), 3-hydroxydecanoate (C_{10}) (32.8 ± 2.8 mol%) and 3-hydroxydodecanoate (C_{12}) (21.0 ± 3.2 mol%). *In vivo* action was studied in a mineral liquid medium without carbon source, and in different buffer solutions with varied pH, molarity, ionic strength (*I*) and temperature. Rate and percentage of *in vivo* depolymerization were highest in 0.2 M Tris-HCl buffer (pH 9, *I* = 0.2 M, 30 ºC) at 0.21 g L^{-1} h^{-1} and 98.6 ± 1.3 wt%, respectively. There is a congruity vis-à-vis to specific buffer type, molarity, pH, *I* and temperature values for superior *in vivo* depolymerization activities. For *in vitro* depolymerization studies, cell-free system was obtained from lysing bacterial cells suspension by ultrasonication at optimum conditions (frequency 37 kHz, 30 % of power output, < 25 ºC for 120 minutes). The comparison between *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA was made. *In vitro* depolymerization showed lower depolymerization rates and activities compared to *in vivo* depolymerization. However, final product yield of *in vitro* depolymerization showed higher yield compared to *in vivo* depolymerization. The monomer liberation rate reflected the mol% distribution of the initial polymer subunit composition, and the resulting direct individual products of depolymerization were identical for both *in vivo* and *in vitro* processes. It points to exo-type reaction for both processes, and potential biological route to chiral molecules.

ABSTRAK

Poli-3-hidroksialkanoat berantai sederhana panjang adalah polimer bolehurai yang dibentuk daripada hidroksialkanoat berbeza dengan konfigurasi-*R* sebagai unit-unit monomer dan adalah sumber bernilai biobahan. Kajian penghasilan, penguraian *in vivo* dan *in vitro* poli-3-hidroksialkanoat berantai sederhana panjang intraselular oleh *Pseudomonas putida* Bet001 daripada asid laurik telah dijalankan. Komposisi polimer (> 50 % jumlah biojisim) dan kepekatan biojisim (8 g L^{-1}) paling tinggi telah diperoleh pada nisbah 20 karbon-kepada-nitrogen, 1 g L^{-1} kepekatan awal sel, dan 48 jam pengeraman. Poli-3-hidroksialkanoat berantai sederhana panjang mengandungi 3-hidroksiheksanoat (C_6) (6.1 ± 2.0 %mol), 3-hidroksioktanoat (C_8) (40.1 ± 2.7 %mol), 3-hidroksidekanoat (C_{10}) (32.8 ± 2.8 %mol) dan 3-hidroksidodekanoat (C_{12}) (21.0 ± 3.2 %mol). Penguraian *in vivo* dijalankan dalam media larutan mineral tanpa sumber karbon dan larutan penimbal berbeza pH, kemoralan, kekuatan ionic (*I*) dan suhu. Kadar dan peratusan penguraian polimer paling tinggi di dalam penimbal Tris-HCl, 0.2 M (pH 9, *I* = 0.2 M, 30 ºC) pada 0.21 g L^{-1} h^{-1} dan 98.6 ± 1.3 %berat, masing-masing. Terdapat kesepadanan 'vis-à-vis' terhadap jenis penimbal tertentu, kemolaran, pH, *I* dan suhu bagi penguraian berkesan. Untuk kajian penguraian *in vitro*, sistem bebas-sel diperolehi daripada pemecahan ampaian sel oleh ultrasonikator pada keadaan optimum (frekuensi 37 kHz, 30 % kuasa, suhu < 25 ºC, selama 120 minit). Perbandingan penguraian antara *in vivo* dan *in vitro* intraselular polimer telah dilakukan. Penguraian *in vitro* menunjukkan kadar dan akitiviti penguraian lebih rendah berbanding penguraian secara *in vivo*. Namun, hasil akhir produk penguraian *in vitro* adalah lebih tinggi daripada hasil produk penguraian *in vivo*. Kadar penguraian monomer mencerminkan taburan subunit asal komposisi polimer dan menghasilkan produk langsung individu telah dikenalpasti untuk kedua-dua penguraian. Ini adalah titik kepada tindak balas penguraian jenis luaran untuk kedua-dua proses, dan berpotensi sebagai kaedah secara biologi untuk menghasilkan molekul kiral.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF SYMBOLS, ABBREVIATIONS AND CHEMICAL FORMULAS

Symbols	Full Name
M_w	Average weight molecular weight
˚C	Celsius
˚C min^{-1}	Celsius per minute
Z	Charge no. of ion
C	Concentration
Da	Dalton
T_d	Decomposition temperature
ΔH_m	Enthalpy of fusion
T_g	Glass transition temperature
g	Gram
g L^{-1}	Gram per liter
g L^{-1} h^{-1}	Gram per liter per hour
g mol^{-1}	Gram per mol
g	Graviti
I	Ionic strength
kDa	Kilodalton
kHz	Kilohertz
kPa	Kilopascal
<	Less than
L	Liter
m/z	Mass-to-charge ratio
T_m	Melting temperature
m	Meter

µL	Microliter
µm	Micrometer
µmol	Micromol
µmol L^{-1}	Micromol per liter
mg	Milligram
mg mL^{-1}	Milligram per milliliter
mL	Milliliter
mL min^{-1}	Milliliter per minute
mm	Millimeter
mM	Millimolar
M	Molar
mol L^{-1}	Mol per liter
M_t	Monomer mass at time, t
>	More than
nm	Nanometer
N	Normality
M_n	Number average molecular weight
ppm	Part per million
A	Peak area
p	P-value, probability of obtaining a result equal to or "more extreme" than what was actually observed, when the null hypothesis is true
%	percent
R_f	Relative response factor
rpm	Rotation per minute
n	Sample size
t	Time

M_0	Total mass of monomer
U	Unit
U L^{-1}	Unit per liter
v/v	Volume per volume
wt%	Weight percent
w/v	Weight per volume
w/w	Weight per weight

Abbreviations	**Full Name**
3HA	3-hydroxyalkanoic acid
3HB	3-hydroxybutyric acid
3HD	3-hydroxydecanoic acid
3HDD	3-hydroxydodecanoic acid
3HHx	3-hydroxyhexanoic acid
3HO	3-hydroxyoctanoic acid
3HV	3-hydroxyvaleric acid
4HB	4-hydroxybutyric acid
*a*PHA	Artificial PHA granule
BET Lab	Bioprocess and Enzyme Technology Laboratory
C	Carbon
C/N	Carbon-to-nitrogen ratio
CoA	Coenzyme A
DI	Deionized water
*d*PHA	Denatured PHA
*d*PHB	Denatured PHB
DCM	Dichloromethane

DSC	Differential scanning calorimetry
DF	Dilution factor
DMSO	Dimethyl sulfoxide
DCW	Dry cell weight
*e*PHA depolymerase	Extracellular PHA depolymerase
FESEM	Field emission scanning electron microscopy
FID	Flame ionization detector
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GPC	Gel permeation chromatography
HMDS	Hexamethyldisilazane
HPLC	High pressure liquid chromatography
HA	Hydroxyalkanoate
IS	Internal standard
*i*PHA depolymerase	Intracellular PHA depolymerases
LCA	Life cycle assessment
lcl-PHA	Long-chain-length PHA
MALDI	Matrix assisted laser desorption ionization
mcl-PHA	Medium-chain-length PHA
MSM	Mineral salts medium
MSM 1	Mineral salts medium after 72 hours biosynthesis in the original medium
MSM 2	Mineral salts medium lacking carbon source and ammonium
MSM 3	Mineral salts medium lacking carbon source with 1 g L^{-1} ammonium
MT	Minimal trace elements

MW	Molar mass
*n*PHA	Native PHA granule
N	Nitrogen
BSTFA	N,N–bis(trimethyl-silyl)trifluoro-acetamide
NMR	Nuclear magnetic resonance
NA	Nutrient agar
NB	Nutrient broth
NR	Nutrient rich
OD	Optical density
PKO	Palm kernel oil
POME	Palm oil mill effluent
PMSF	Phenylmethylsulfonyl fluoride
P(3H10UD-*co*-3H8N-*co*-3H6P)	Poly(3-hydroxy-10-undecenoate-*co*-3-hydroxy-8-nonenoate-*co*-3-hydroxy-6-heptenoate)
PHPV	Poly(3-hydroxy-5-phenylvalerate)
PHB/P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-valerate)
PHD	Poly(3-hydroxydecanoate)
PHDD	Poly(3-hydroxydodecanoate)
PHHp	Poly(3-hydroxyheptanoate)
PHHx	Poly(3-hydroxyhexanoate)
P(3HHx-*co*-3HO)	Poly(3-hydroxyhexanoate-*co*-3-hydroxyoctanoate)
PHN	Poly(3-hydroxynonanoate)
PHO	Poly(3-hydroxyoctanoate)
PHPp	Poly(3-hydroxypropionate)

PHUD	Poly(3-hydroxyundecanoate)
P(3HUD-*co*-3HN-*co*-3HP)	Poly(3-hydroxyundecanoate-*co*-3-hydroxynonanoate-*co*-3-hydroxyheptanoate)
PHV	Poly(3-hydroxyvalerate)
P(4HB)	Poly(4-hydroxybutyrate)
PDI	Polydispersity index (M_w/M_n)
PHA	Polyhydroxyalkanoates
PLA	Polylactic acid
PDLA	Poly[(*R*)-lactic acid]
PLLA	Poly[(*S*)-lactic acid]
PTFE	Polytetrafluoroethylene
P. putida Bet001	*Pseudomonas putida* Bet001
BDH	(*R*)-3HB dehydrogenase
(*R*)-3HA	(*R*)-3-hydroxyalkanoic acids
(*R*)-3HB	(*R*)-3-hydroxybutyric acids
SPKO	Saponified palm kernal oil
SEM	Scanning electron micrographs
scl-PHA	Short-chain-length PHA
SDS	Sodium dodecyl sulfate
spp	Species
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TGA	Thermogravimetric analysis
TCA	Tricarboxylic acid
USA	United States of America
UV/VIS	Ultraviolet–visible
Vol/ *V*	Volume

Chemical Formulas	Full Name
N-H	Amine group
NH_4^+	Ammonium cation
$NaNH_4HPO_4.4H_2O$	Ammonium sodium phosphate dibasic tetrahydrate
$(NH_4)_2SO_4$	Ammonium sulfate
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CO_2	Carbon dioxide
-COOH	Carboxylic group
H^+	Cationic form of atomic hydrogen
$CoCl_2.6H_2O$	Cobalt (II) chloride hexahydrate
$CuCl_2.2H_2O$	Copper (II) chloride dihydrate
$C_{16}H_{36}O_3Si_2$	Decanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
$CDCl_3$	Deuterated chloroform
K_2HPO_4	Dipotassium phosphate
Na_2HPO_4	Disodium hydrogen phosphate
$C_{18}H_{40}O_3Si_2$	Dodecanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
$C_{12}H_{28}O_3Si_2$	Hexanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
HCl	Hydrochloric acid
OH^-	Hydroxide anion
-OH	Hydroxyl group
$FeSO_4.7H_2O$	Iron (II) sulfate heptahydrate
$C_{12}H_{24}0_2$	Lauric acid
$MgCl_2$	Magnesium chloride
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate

$MnCl_2.4H_2O$	Manganese (II) chloride tetrahydrate
NADPH	Nicotinamide adenine dinucleotide phosphate
$C_{14}H_{32}O_3Si_2$	Octanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
Na_2SO_4	Sodium sulfate
H_2O	Water
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Biodegradable polymers are defined as degradable materials in which the degradation results from the action of microorganisms and ultimately the material is converted to water, carbon dioxide (in the case of aerobic degradation) and/or methane (in the case of anaerobic degradation) and a new cell biomass (Rizzarelli & Carroccio, 2014). Polyhydroxyalkanoates (PHAs) are one such example because they can undergo biodegradation under various environmental conditions (Jendrossek, 2007; Tokiwa & Ugwu, 2007). It is a family of biopolyesters with diverse structures, and synthesized by microorganisms as reserve granules. It is considered as an environmental-friendly and sustainable alternative, especially in the face of rapid consumption of non-renewable fossil-based resources (De Eugenio et al., 2007; Chen, 2010). The potential of PHA is not limited to the versatility and flexibility of the neat polymer but also its biodepolymerization products such as monomers, dimers and oligomers, which provide a new route to the synthesis of platform chemicals.

More than 150 different types of hydroxyalkanoates have been identified as monomer constituents resulting in exceptionally diverse structures of PHA (Chen & Wu, 2005; Chen, 2009; Jendrossek, 2009). In PHA, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer. Each monomer contains the chiral carbon with (*R*)-stereochemical configuration (De Eugenio et al., 2007; Sin et al., 2010). (*R*)-3-hydroxyalkanoic acids ((*R*)-3HAs) produced by the hydrolysis of PHAs can be used as chiral starting materials in fine chemicals, pharmaceutical and medical industries (Chen, 2010). Nowadays, chiral PHA monomers can be produced by depolymerizing the biosynthesized, accumulated PHA using different ways (Chen, 2009). Methods for producing (*R*)-3HAs by chemical digestion have been reported. However, large amounts of organic solvents are needed for the process in

addition to low production efficiency and multi-step requirements thus making the biological route for (*R*)-3HAs production to be potentially more viable (Ren et al., 2005; Ruth et al., 2007).

Researches on the biodegradation of PHA should clearly distinguish between intracellular- and extracellular PHA depolymerizations (Jendrossek & Handrick, 2002; Jendrossek, 2007). Intracellular depolymerization is the hydrolysis of an endogenous carbon reservoir by the accumulating bacteria themselves, while extracellular depolymerization is the utilization of an exogenous carbon source not necessarily by the accumulating microorganisms alone but also other organisms (Handrick et al., 2004a; Tokiwa & Calabia, 2004). Intracellular- and extracellular PHA exist in different conformations. Intracellular native PHA (*n*PHA) granules exhibit amorphous, rubbery state. *n*PHA consists of highly mobile chains of the carbon backbone giving it a disordered conformation in addition to being covered by a dense layer consisting of mainly protein (phasins, PHA synthase, PHA depolymerase and other proteins). Upon extraction from the cell, or after cell lysis or death, the surface layer of *n*PHA granules is rapidly damage or lost, and the PHA chain tends to adopt an ordered helical conformation and develop a crystalline phase known as denatured PHA (*d*PHA) (Handrick et al., 2004b; Gebauer & Jendrossek, 2006; Jendrossek, 2007; Uchino et al., 2007; Chen et al., 2009). Extracellular depolymerization of PHA film has been well-studied and characterized in terms of biochemical and biological properties compared to intracellular *n*PHA depolymerization (Yoon & Choi, 1999).

Intracellular PHA depolymerization could occur as *in vivo* and *in vitro* processes. *In vivo* depolymerization of intracellular PHA occurs on the PHA granule where intracellular PHA depolymerases (*i*PHA depolymerase) is also located, and the activity is associated with the protein complex encompassing the *n*PHA inclusion bodies inside the bacterial cells. Degradation takes place without isolation and purification of the *i*PHA

depolymerases enzyme and *n*PHA as a substrate (Foster et al., 1996; Ren et al., 2005; De Eugenio et al., 2007). On the other hand, *in vitro* depolymerization of intracellular PHA occurs when the polymer granules are isolated from intact bacterial cell with their protein coat intact without losing their amorphous and elastomeric nature characteristic, and the *i*PHA depolymerase remains active on the isolated PHA inclusion bodies (Foster et al., 1996; Stuart et al., 1996; Foster et al., 1999; Handrick et al., 2004a).

Currently, there is a strong demand in the chemical industries for chiral molecules from renewable resources. Depolymerization of PHA to produce chiral molecules in the form of monomers, dimers and oligomers is envisaged as a potential environmental-friendly route to platform chemicals with high yield and productivity and at a relatively low cost. The main goal of this research is to develop an efficient method to produce depolymerization products of mcl-PHA in the form of (*R*)-3HAs. The mcl-PHA feedstock will be produced by *Pseudomonas putida* Bet001 from a renewable resource such as palm oil fatty acids. In addition, the study seeks to investigate and understand the fundamental aspect biosynthesis, *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA in *P. putida* Bet001 as a model organism.

1.1.1 *In vivo* depolymerization

In *in vivo* depolymerization study, PHA producer microorganism will be cultured in a medium with excess carbon source until the late PHA accumulation phase or stationary growth phase when carbon source is depleted (Ren et al., 2005). Then, bacterial cells containing PHA will be transferred into a medium lacking carbon substrate to systematically investigate *in vivo* depolymerization of PHA under defined conditions. Under specific conditions, the living cells will degrade and exploit the accumulated PHA granules using *i*PHA depolymerase to form monomers or possibly oligomers for their survival (Lee et al., 1999; Tokiwa & Ugwu, 2007). Nevertheless, it is important to

develop suitable conditions to prevent the bacterial cells from completely consuming the individual hydroxycarboxylic acids after internal hydrolysis of PHA granules.

Recently, researches on intracellular depolymerization of PHA in short-chain-length PHA (scl-PHA) producers have increased significantly while similar studies in medium-chain-length PHA (mcl-PHA) producers are scarce. Thus, biosynthesis and *in vivo* depolymerization of intracellular mcl-PHA that contains four different chiral monomers *viz.* 3-hydroxyhexanoates (3HHx), 3-hydroxyoctanoates (3HO), 3-hydroxydecanoates (3HD) and 3-hydroxydodecanoates (3HDD) in *P. putida* Bet001 were investigated in this study. Preference of monomer fraction and identity by intracellular depolymerization activities during the *in vivo* process will be studied alongside selected process parameters. Identification and quantification of extracellular products directly arising from *in vivo* depolymerization of mcl-PHA were also carried out.

1.1.2 *In vitro* depolymerization

Recently, various enantiomerically pure (*R*)-3HAs have been produced by *in vivo* depolymerization of intracellular PHA since *in vitro* depolymerization of intracellular PHA is a tedious method with significant difficulties in *n*PHA isolation, and purification of *i*PHA depolymerase enzyme without losing *n*PHA amorphous characteristic and enzyme activity. Isolated *n*PHA granules and *i*PHA depolymerase enzyme are extremely sensitive to chemical and physical stresses (Yoon & Choi, 1999; Jendrossek, 2007). Despite the limitations, it is possible to obtain higher depolymerization rate for *in vitro* depolymerization of intracellular *n*PHA compared to *in vivo* as in scl-PHA. In this study, *in vitro* depolymerization of intracellular mcl-PHA was investigated using cell-free biology system. Cell-free biology involves the activation of complex biological processes without using living cells (Swartz, 2006; Swartz, 2012). The cells are opened up (lysed)

and unpurified portions are used. In cell-free technologies, source cells are grown, harvested, and then lysed. The lysate can be used directly or centrifuged to remove suspended solids and further processed, if needed (Swartz, 2012). For the purpose of current investigation, crude preparation of lysed cell components was used without further purification nor pre-treatment. By exploiting PHA machinery of *P. putida* Bet001 as a model system for mcl-PHA depolymerization process, a comparison between *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA was also made.

1.2 Objectives of Study

1.2.1 Biosynthesis of intracellular mcl-PHA studies

i. To study the growth and mcl-PHA biosynthesis by *P. putida* Bet001 in different carbon sources;

ii. To produce high mcl-PHA content in the bacterial cell under favourable conditions using the most suitable carbon source;

1.2.2 *In vivo* depolymerization of intracellular mcl-PHA studies

i. To study *in vivo* depolymerization of intracellular mcl-PHA in appropriate medium

a) in production medium (mineral medium)

b) in buffer solution;

ii. To determine the depolymerization activity, monomer preference and rate of *in vivo* depolymerization of intracellular mcl-PHA;

iii. To analyse and characterize exogenous direct products from *in vivo* depolymerization of intracellular mcl-PHA;

1.2.3 Comparison of *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA studies

i. To develop cell-free biology system for *in vitro* depolymerization of intracellular mcl-PHA using lysed cell from ultrasound disruption technique;

ii. To compare the depolymerization activity, monomer preference and rate between *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA;

iii. To compare, analyse and characterize exogenous direct products from *in vivo* and *in vitro* depolymerizations of mcl-PHA.

CHAPTER 2: LITERATURE REVIEW

2.1 Polyhydroxyalkanoates (PHA)

Polymers are divided into two main groups i.e. synthetic polymers (usually non-biodegradable) such as polystyrene, polyethylene, nylon; and natural polymers (biodegradable) such as proteins, cellulose, silk (Ghosh et al., 2013; Panchal et al., 2013). At present, it is estimated that over 200 million tons of synthetic polymers derived from petrochemicals are being produced globally per year, and the production is still increasing (Ghosh et al., 2013; Sun et al., 2014). The consumption of conventional petroleum-based plastics has become a necessity in our daily life and far exceeds its recyclability because of their versatilities for a wide range application, low production costs, high durability, outstanding technical properties and easy to obtain (Panchal et al., 2013; Martínez et al., 2014; Rizzarelli & Carroccio, 2014; Urtuvia et al., 2014). Consequently, elevated production of plastic materials results in the increase of oil consumption, waste accumulation problems, dumping of solid plastic wastes in the sea and river, depletion of fossil resources due to their persistence in the environment, greenhouse gas emissions and ozone layer depletion due to plastic burning that usually produce some noxious gases like furans and dioxins (Ojumu et al., 2004; Daly et al., 2005; Ghosh et al., 2013; Rizzarelli & Carroccio, 2014). These problems have stimulated a deep interest in natural polymers as an ecologically useful alternative to synthetic plastics (Ojumu et al., 2004; Rizzarelli & Carroccio, 2014).

Natural or biodegradable polymers are defined as materials that are able to completely degrade into carbon dioxide and water in aerobic conditions, methane in anaerobic conditions, and as well as into a new cell biomass by the action of naturally occurring microorganisms, such as bacteria, fungi and algae (Ojumu et al., 2004; Rizzarelli & Carroccio, 2014; Iwata, 2015). There are several types of biodegradable polymers such as cellulose, starch, polylactic acid (PLA), poly[(*S*)-lactic acid] (PLLA),

poly[(*R*)-lactic acid] (PDLA), polyester-amide, polysaccharides and also polyhydroxyalkanoates (PHA), which have received much attention in recent years (Ojumu et al., 2004; Panchal et al., 2013). PHA are targeted for uses in various areas such as in industrial, agricultural, food packaging and biomedical applications due to their biodegradability, biocompatibility, resorbability, piezoelectricity, thermoplasticity, hydrophobicity, elastomericity, enantiomerically pure and non-toxicity characteristics as well as becoming a practical solution in coping with the plastic and waste disposal problems, reducing greenhouse gas emissions and oil dependency (Daly et al., 2005; Thakor et al., 2005; Gumel et al., 2012; Ghosh et al., 2013; Panchal et al., 2013; Sun et al., 2014; Kanmani et al., 2016). PHA also garnered attention because they can be readily produced from renewable resources (such as sugars, fatty acids and plant oil), raw materials by aerobic synthesis and from waste management by composting and anaerobic digestion (Tokiwa & Ugwu, 2007; Urtuvia et al., 2014). Life cycle assessment (LCA) indicates that the energy requirements for PHA production are lower than the energy requirements for conventional high density plastics because it can be synthesized from renewable low-cost feedstocks, polymerization operated under mild process conditions and can be biodegraded in the environment without forming any toxic products (Ghosh et al., 2013; Urtuvia et al., 2014).

Numerous bacteria have been found to produce PHA as the intracellular carbon and energy storage material when one of the nutritional elements, such as nitrogen, phosphorus, oxygen, potassium, or sulfur, is being limited while the carbon source is in excess (Lee et al., 1999; Ren et al., 2005; Kim et al., 2007; Tokiwa & Ugwu, 2007; Chen et al., 2009; Gumel et al., 2012; Urtuvia et al., 2014). Stored PHA can be depolymerized by intracellular depolymerases into monomers and can be reutilized by the bacteria as a carbon and energy source if the cells are under carbon starvation (Ojumu et al., 2004; Ren et al., 2005; Ruth et al., 2007). Over 250 different bacteria, including Gram-negative and

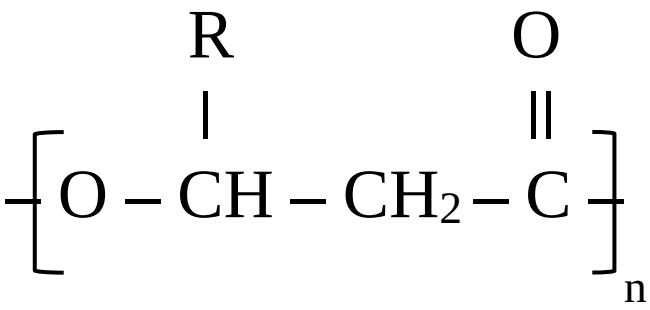
Gram-positive species, have been reported to accumulate various PHA such as *Cupriavidus necator*, *Bacillus* spp., *Alcaligenes* spp., *Pseudomonas* spp., *Aeromonas hydrophila*, *Rhodopseudomonas palustris*, *Escherichia coli*, *Burkholderia sacchari* and *Halomonas boliviensis* (Ojumu et al., 2004; Panchal et al., 2013).

PHA can be divided into three broad groups based on the number of carbon atoms in the monomer units; the short-chain-length PHA (scl-PHA) contains 3 to 5 carbons atoms, medium-chain-length PHA (mcl-PHA) comprises of 6 to 14 carbons atoms, and long-chain-length PHA (lcl-PHA) consists of more than 14 carbon atoms in the monomer units (Ojumu et al., 2004; Ren et al., 2005; Chung et al., 2011; Sin et al., 2011a; Martínez et al., 2014; Urtuvia et al., 2014). Generally scl-PHA are thermoplastics, stiff, brittle and possess a high degree of crystallinity whereas mcl-PHA are elastomers, sticky, amorphous materials with a low degree of crystallinity and a low melting temperature (Kim et al., 2007; Philip et al., 2007; Timbart et al., 2007; Chung et al., 2011; Panchal et al., 2013). Hybrid PHA copolyesters are expected to have a wide variety of mechanical properties ranging from hard crystalline to elastic and combination of both scl-PHA and mcl-PHA monomer units in the polymer conceivably enhanced the representative PHA to be superior and flexible material depending on the mol percentage of their constituents and consequently broaden the potential applications of PHA (Lee, 2005; Kim et al., 2007; Philip et al., 2007; Sin et al., 2010; Razaif - Mazinah et al., 2016).

The nature, composition, properties, chemical characteristics, and proportion of the PHA monomers are varied and influenced by the type of carbon sources supplied, the type of microorganisms used, media ingredients, the culture conditions provided and the modes of fermentation (Ojumu et al., 2004; Keshavarz & Roy, 2010; Panchal et al., 2013; Urtuvia et al., 2014). Recently, several strategies have been developed to improve conditions for efficient and cost effective PHA production which involve genetic engineering, potential bacterial species, sourcing cheap and renewable carbon energy in

order to obtain high productivity, and high recovery efficiency of PHA (Ojumu et al., 2004; Salim et al., 2012; Panchal et al., 2013).

PHA are a family of linear, head-to-tail polyesters composed of several units of chiral 3-hydroxy fatty acid monomers linked to each other through ester linkages (Figure 2.1) (Philip et al., 2007; Prieto et al., 2007; Panchal et al., 2013). Appreciable number of PHA with over 150 different chiral hydroxy fatty acid monomers have been found to be incorporated into the polymer with molecular masses ranging from 50,000 to 1,000,000 Da (Foster et al., 1996; Yoon & Choi, 1999; Handrick et al., 2004a; Ren et al., 2005; Martínez et al., 2014). All of the monomeric units of PHA are enantiomerically pure and in the (*R*)-configuration. (*R*)-hydroxyalkanoic acids produced by the hydrolysis of PHA can also be widely used as chiral starting materials in fine chemicals, pharmaceutical and medical industries (Philip et al., 2007). Conversely, the high molecular weight of PHA is unsuitable for certain uses such as the design of special polymers for application in biodegradable nanoparticles (Kanmani et al., 2016). Therefore, the potential application of PHA is not limited to the versatility and flexibility of the neat polymer but also its depolymerization products such as monomers, dimers and oligomers, which provide a new route to the synthesis of platform chemicals. Degradation or depolymerization of PHA, like other polyesters, can occur *via* one or several mechanisms including enzymatic hydrolysis, thermal decomposition and chemical decomposition or hydrolysis (Yu et al., 2005). The PHA depolymerization can be divided into two conditions; intracellular depolymerization (enzymatic) and extracellular depolymerization (enzymatic, chemical and thermal) (Figure 2.2).



***R* Group**	**Carbon no.**	**PHA Polymer**
Hydrogen	C_3	Poly(3-hydroxypropionate), PHPp
Methyl	C_4	Poly(3-hydroxybutyrate), PHB
Ethyl	C_5	Poly(3-hydroxyvalerate), PHV
Propyl	C_6	Poly(3-hydroxyhexanoate), PHHx
Butyl	C_7	Poly(3-hydroxyheptanoate), PHHp
Pentyl	C_8	Poly(3-hydroxyoctanoate), PHO
Hexyl	C_9	Poly(3-hydroxynonanoate), PHN
Heptyl	C_{10}	Poly(3-hydroxydecanoate), PHD
Octyl	C_{11}	Poly(3-hydroxyundecanoate), PHUD
Nonyl	C_{12}	Poly(3-hydroxydodecanoate), PHDD
Decyl	C_{13}	Poly(3-hydroxytridecanoate)
Undecyl	C_{14}	Poly(3-hydroxytetradecanoate)

Figure 2.1: General chemical structure of polyhydroxyalkanoates.

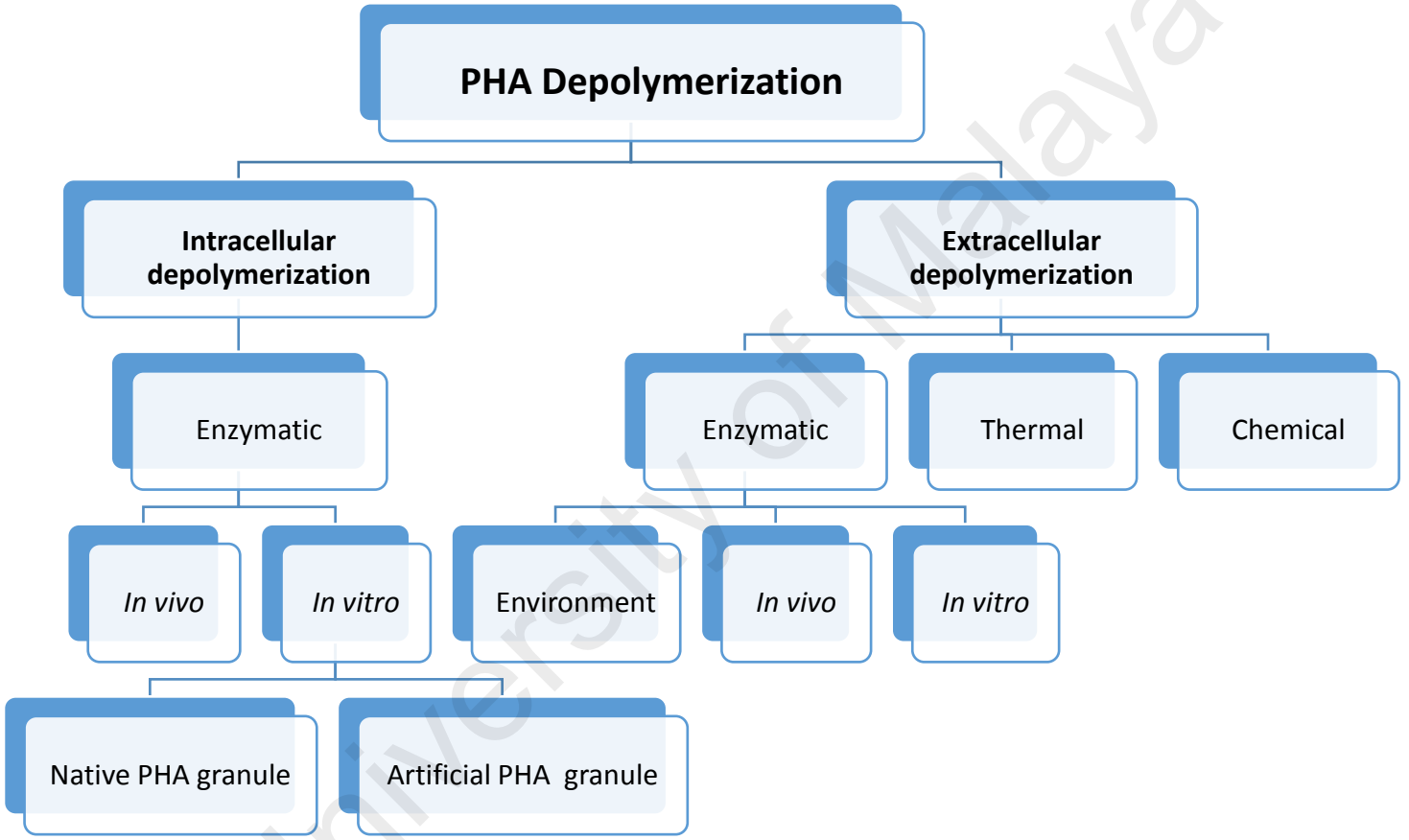


Figure 2.2: Different types of PHA depolymerizations.

2.2 Biosynthesis and Biodepolymerization of PHA

Depending on the microorganism and fermentation strategy, PHA can be accumulated at up to 90 % of cellular dry weight during unbalanced growth in the form of inclusion bodies (Handrick et al., 2004a; Prieto et al., 2007; Shah et al., 2008). In the PHA biosynthetic pathway of microbial cells, provision, polymerization of hydroxyalkanoate (HA) monomers involve various type of enzymes (Chuah et al., 2013). In the scl-PHA biosynthesis pathway, carbohydrates that are unrelated in structure to the final PHA monomer are used by *Alcaligenes eutrophus* or *Cupriavidus necator*, which harbors the well-studied pathway for the PHA biosynthesis. Three key enzymes are involved i.e. *β*-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase, and PHA synthase. Poly(3-hydroxybutyrate) (P(3HB)) for instance, is synthesized in a three-step reaction in excess carbon available starting with acetyl-CoA. The first enzyme, in the pathway *β*-ketothiolase promotes the condensation of two acetyl CoA moieties in a reversible manner from the tricarboxylic acid (TCA) cycle to form acetoacetyl-CoA. Then, acetoacetyl-CoA is reduced to (*R*)-3-hydroxybutyrl-CoA by NADPH-dependent acetoacetyl-CoA reductase. Finally, the PHA synthase enzyme catalyses the polymerization *via* esterification of 3-hydroxybutyryl-CoA into P(3HB) (Ojumu et al., 2004; Pötter & Steinbüchel, 2005; Philip et al., 2007; Prieto et al., 2007; Tokiwa & Ugwu, 2007; Panchal et al., 2013).

On the other hand, mcl-PHA biosynthesis in bacteria is closely linked to three different metabolic routes that generate mcl-PHA precursor molecules: (i) *de novo* fatty acid biosynthesis pathway, that produces (*R*)-3-hydroxyacyl-CoA precursors from structurally unrelated carbon sources such as glucose, sucrose, fructose and gluconate. This pathway is having a specific importance for it helps generate monomers for PHA synthesis from simple and inexpensive carbon sources; (ii) fatty acid degradation by *β*-oxidation, which is the main metabolic route of fatty acids. When fatty acid is fed to mcl-

producer microorganism, it passes through *β*-oxidation biosynthetic pathway to produce the PHA intermediates thereby losing two carbon atoms per each cycle. In this case, the resulting monomers in the polymer chain were similar in structure to the carbon source or shortened by 2, 4 or 6 carbon atoms, (iii) chain elongation, in which acyl-CoA is extended with acetyl-CoA (Kim et al., 2007; Philip et al., 2007; Prieto et al., 2007; Ren et al., 2009; Gumel et al., 2012; Panchal et al., 2013).

When bacteria are cultured under limited carbon supply or the environment becomes more hospitable, the accumulated PHA can be depolymerized to the corresponding monomers which are reutilized by the cells as carbon and energy sources (Ren et al., 2005; Prieto et al., 2007). In addition to the pathway for PHA synthesis, PHA depolymerization pathway plays an important role on the overall PHA metabolism and can operate simultaneously in bacteria (Stuart et al., 1996; Gao et al., 2001). Depolymerization of PHA is performed in bacteria by at least two different pathways, one involves an intracellular depolymerization process, and also extracellularly (Handrick et al., 2004a; Prieto et al., 2007; Shah et al., 2008).

PHB has been widely used as a model to investigate intracellular PHA depolymerization. Usually, PHB is first depolymerized into 3-hydroxybutyrate (3HB) monomers or 3HB oligomers (e.g., 3HB dimers, 3HB trimers, and 3HB tetramers) by PHB depolymerase. The 3HB oligomers are then hydrolyzed into 3HB by 3HB oligomer hydrolase. The depolymerized products could be further metabolized by the cells to acetoacetate by (*R*)-3HB dehydrogenase (BDH) (Lee et al., 1999; Tokiwa & Ugwu, 2007; Lu et al., 2014). A dehydrogenase acts on the latter and oxidises it into acetylacetate, and a *β*-ketothiolase acts on acetylacetate to break it down into acetyl-CoA. The *β*-ketothiolase enzyme plays an important role in both biosynthetic and depolymerization pathways. Under aerobic conditions, the acetyl-CoA enters the citric acid cycle and is oxidized to carbon dioxide (CO_2) (Philip et al., 2007).

In extracellular depolymerization, individual PHA are much too large to be transported directly across the bacterial cell wall. Therefore, bacteria must have evolved some extracellular hydrolases that are capable in converting the polymers into corresponding hydroxyl acid monomers. The monomers are water soluble and small enough to passively diffuse through the cell wall, where they are being metabolized by *β*-oxidation and TCA cycle to produce CO_2 and water under aerobic conditions, and methane under anaerobic conditions (Shah et al., 2008). The general biosynthesis and depolymerization pathway of PHA is shown in Figure 2.3.

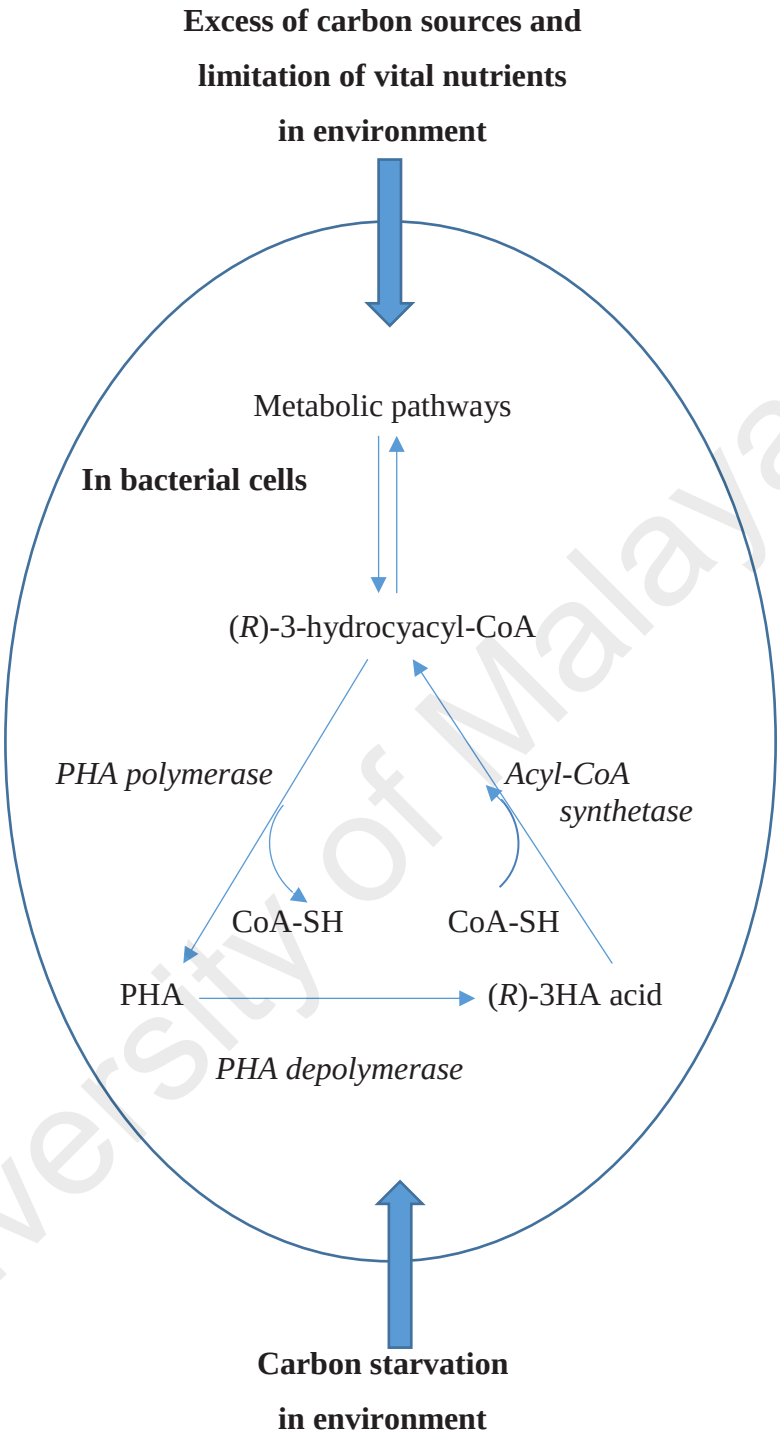


Figure 2.3: Metabolic pathways involved in the synthesis and depolymerization of PHA (Ren et al., 2005).

2.3 Intracellular Depolymerization of PHA

Intracellular depolymerization of PHA occurs when the PHA is in an amorphous "rubbery" state with highly mobile chains and in disordered conformation of the carbon backbone i.e. in a native PHA (*n*PHA) state, a term coined by Merrick and Doudoroff, (1964). The surface of *n*PHA is surrounded with a layer consisting of phospholipids and granule associated proteins (Merrick & Doudoroff, 1964; Lee & Choi, 1999; Handrick et al., 2001; Handrick et al., 2004a; Tokiwa & Calabia, 2004; Pötter & Steinbüchel, 2005; Jendrossek, 2007; Chen et al., 2009). Intracellular inclusion bodies represent a highly organized subcellular assembles and enclosed system in a para-crystalline network of polyester, enzymatic and structural protein, and phospholipid that are responsible for synthesis, depolymerization and containment of PHA (Foster et al., 1996; Stuart et al., 1996; Foster et al., 1999; Sznajder & Jendrossek, 2011). However, a more recent postulate suggests that the surface layer of *n*PHA is generally free of phospholipids. Instead, *n*PHA harbours a considerable number of proteins thus itself representing a supramolecular complex with specific functions rather than as merely storage material (Jendrossek & Pfeiffer, 2014; Bresan et al., 2016). Intracellular PHA, which is accumulated as storage granule in bacterial cell can be depolymerized by several hydrolases and esterases. However, they do not hydrolyze *n*PHA because the enzymes are either located in different cell compartment and do not bind to the polymer because *n*PHA are shielded by the phasin layer. The main enzymes that catalyze the depolymerization of *n*PHA are known as intracellular PHA depolymerases (*i*PHA depolymerase) (Prieto et al., 2007; Jendrossek, 2009). In nature, PHA intracellular depolymerization process comprises the hydrolysis of the endogenous storage material (*n*PHA). The carbon/energy reservoir is mobilized by the *i*PHA depolymerase, later accumulated by microorganisms itself when supply of limiting nutrient is restored (Thakor et al., 2005; Prieto et al., 2007). Nowadays, several

methods have been developed to investigate the intracellular depolymerization of PHA and can be classify into two conditions *viz. in vivo* or *in vitro.*

2.3.1 *In vivo*

In vivo intracellular depolymerization of PHA is an active mobilization (hydrolysis) of an endogenous carbon or energy storage reservoir within the accumulating bacterium itself by intracellular hydrolysis enzyme during the period of starvation once the exogenous carbon source is exhausted (Stuart et al., 1996; Handrick et al., 2001; Jendrossek, 2002; Handrick et al., 2004a; Gebauer & Jendrossek, 2006; Papageorgiou et al., 2008). PHA in their granule is water insoluble, and in the form of discrete inclusion bodies when it is maintained in an osmotically inert state (Foster et al., 1996; Foster et al., 1999). *In vivo* intracellular process, depolymerization of PHA occurs on the PHA granule itself that also locates *i*PHA depolymerase which associated with the protein complex encompassing the *n*PHA inclusion bodies inside the bacterial cells (Foster et al., 1996; Ren et al., 2005; de Eugenio et al., 2007). *In vivo* intracellular depolymerization study of PHA took place without isolation and purification of the *i*PHA depolymerase enzyme and *n*PHA substrates (Yoon & Choi, 1999).

In *in vivo* depolymerization, PHA producer is cultured in medium with excess carbon source until the late phase of PHA accumulation or stationary growth phase, when carbon source is depleted (Ren et al., 2005). Then, the bacterial cells containing PHA are collected and transferred into a medium without carbon substrate to systematically study the *in vivo* depolymerization of PHA under defined condition. Under specific conditions, the living cells depolymerized and utilized the accumulated PHA granules by *i*PHA depolymerase to form monomers or possibly oligomers for their survival (Lee et al., 1999; Tokiwa & Ugwu, 2007; Chen et al., 2009). Under such situation, the cells decrease the concentration of polymerase while increasing the concentration of *i*PHA depolymerase.

There are variations in the protein concentration in the PHA granules because the cells must prepare themselves to mobilize the stored intracellular reserves in order to continue surviving once the carbon source in the media is exhausted (Stuart et al., 1996). For instance, high yield of depolymerization product can be produced in naturally PHA producing bacteria by providing the environmental condition in which cells possess high activity of *i*PHA depolymerase (Lee et al., 1999; Tokiwa & Ugwu, 2007). In an attempt to increase the activity of *i*PHA depolymerase, Yoon and Choi (1999) proposed to increase the content of total protein in cells by the addition of nitrogen source in the depolymerization medium. Minimal depolymerization process that was observed in the absence of nitrogen may be due to the metabolic suppression that prevents the flow of carbon derived from PHA storage, hence the limited amount of *i*PHA depolymerase (Yoon & Choi, 1999). The depolymerization products that are excreted into the medium can be further isolated and used as valuable natural chiral compound products. Hence, it is important to develop suitable conditions to prevent the bacterial cells to completely utilize the individual hydroxycarboxylic acids after depolymerizing the PHA (Lee et al., 1999; Tokiwa & Ugwu, 2007; Chen et al., 2009). The depolymerization products are metabolized to acetoacetate by the cells *via* the action of (*R*)-3HB dehydrogenase. On the other hand, the method is suitable for determining the compositional and microstructural changes that occurred during *in vivo* intracellular PHA depolymerization (Lee et al., 1999; Yoon & Choi, 1999; Tokiwa & Ugwu, 2007).

In other studies, a process has been reported whereby continuous limitation of the carbon source and nutrients in an anaerobic condition inhibits the cells from metabolizing the monomers from intracellular PHA depolymerization by lowering the concentration of (*R*)-3-hydroxyalkanoates dehydrogenase, which in turn will help to minimize the conversion of 3-hydroxyalkanoic acid (3HA) to acetoacetate (Lee et al., 1999; Kawata et al., 2012; Roy & Visakh, 2015). Others identified factors that contribute to *in vivo*

depolymerization are substrate concentration, working temperature, extracellular pH and ionic content of the media (Foster et al., 1999; Ren et al., 2005; Tokiwa & Ugwu, 2007; Roy & Visakh, 2015). Unfortunately, assaying *i*PHA depolymerase is difficult, due to the nature of the polymeric substrates as mentioned earlier namely the *n*PHA are amorphous and harbour a considerable number of proteins on their polymer surface (Jendrossek, 2009; Jendrossek & Pfeiffer, 2014). Table 2.1 shows the different types of bacteria according to their particular types of PHA produced, depolymerization conditions and depolymerization efficiencies for *in vivo* depolymerization of intracellular PHA.

Table 2.1: *In vivo* depolymerization of intracellular PHA by various bacteria.

Microorganisms	Depolymerization conditions	Type of PHA	Efficiency of PHA depolymerization	References
Streptomyces sp. JM3	50 mM phosphate buffer, pH 10, 30 ˚C, 100 rpm	HO, HD, HDD, HOD, HXD	Not stated	(Allen et al., 2012)
Halomonas sp. KM-1	Modified SOT medium, 33 °C, under microaerobic conditions, 16.4 g L^{-1} PHB	PHB	92.7 wt% after 18 hours	(Kawata et al., 2012)
Pseudomonas putida GPo1	50mM phosphate buffer, pH 10, 30 ˚C, 1.5 g L^{-1} PHA	P(3Hhx-*co*-3HO), P(3HUD-*co*-3HN-*co*-3HP), P(3H10UD-*co*-3H8N-*co*-3H6P)	70 wt% after 8 hours	(Ruth et al., 2007)
Pseudomonas putida GPo1	50mM phosphate buffer, pH 11, 30 ˚C, without shaking, 0.48 g L^{-1} PHA	P(3Hhx-*co*-3HO)	90 wt% after 9 hours	(Ren et al., 2005)
Alcaligenes latus	In water with pH 4, 37 ˚C, without shaking, 10.9 g L^{-1} PHB	PHB	96 wt% after 30min	(Lee et al., 1999)

Table 2.1: continued.

Microorganisms	Depolymerization conditions	Type of PHA	Efficiency of PHA depolymerization	References
Pseudomonas oleovorans	In water with pH 7, 30 °C, without shaking, 15 g L^{-1} PHA	Mcl-PHA (C_6-C_{10})	10.3 wt% after 4 days	(Lee et al., 1999)
Pseudomonas aeruginosa	In water with pH 7, 30 °C, without shaking, 17 g L^{-1} PHA	Mcl-PHA (C_6-C_{12})	8.5 wt% after 4 days	(Lee et al., 1999)
Ralstonia eutropha	In water with pH 7, 30 °C, without shaking, 27.4 g L^{-1} PHA	PHB/V	23.4 wt% after 35 h	(Lee et al., 1999)
Hydrogenophaga pseudoflava	Carbon-free mineral medium containing 1 g L^{-1} ammonium sulphate,35 °C, shaking at 190 rpm, 2.5 g L^{-1} P3HB	P3HB, P(3HB-*co*-4HB), P(3HB-*co*-3HV)	P3HB 95 wt%, 3HB-4HB 76 wt%, , 3HB-3HV 76 wt% after 60 hours, no depolymerization for 4HB	(Yoon & Choi, 1999)

2.3.2 *In vitro*

In vitro depolymerization of intracellular PHA occurs when the *n*PHA granules are isolated from whole bacterial cell with their protein coat intact without losing their amorphous and elastomeric nature characteristics. The *i*PHA depolymerase remains active on isolated PHA inclusion bodies. There are two conditions pertaining to *i*PHA depolymerase in *in vitro* depolymerization of intracellular PHA. Firstly, *i*PHA depolymerase associated with the *n*PHA granules with the enzymatic hydrolysis activity of the polymer within this cell free system occurs in association with the organized protein lattice that encompasses the stored polymer (Foster et al., 1996; Foster et al., 1999). Secondly, *i*PHA depolymerase released from *n*PHA granules once the *i*PHA depolymerase is solubilized from the granule by specific treatment such as freeze thawing. The *i*PHA depolymerase itself remained enzymatically active in the supernatant since it has been demonstrated that the preparation enhance the rate of polymer granule depolymerization when added to other granule suspensions or freshly isolated polymer granules. Likewise, when colloidal suspensions of purified, amorphous PHA were placed in contact with that depolymerase, they also underwent rapid depolymerization (Foster et al., 1996; Stuart et al., 1996).

The different ionic contents of the media and inclusion body polymer-to-buffer ratio can significantly affect the rate of enzymatic action of inclusion body depolymerization (Foster et al., 1996; Foster et al., 1999). The depolymerization of *n*PHA requires high ionic concentration for strong activity. Previous study reported that the presence of magnesium and sodium helped to increase of *n*PHA granule depolymerization. However, the addition of small quantities of inhibitors such as Triton-X-100, phenylmethylsulfonyl fluoride (PMSF) and others will inactivate the depolymerization enzymes by interacting with serine residues within their active sites (Foster et al., 1996). Recently, *i*PHA depolymerase activity could be demonstrated *in*

vitro using radiolabeled substrates or by pH stat for mcl-*n*PHA and scl-*n*PHA, respectively. Unfortunately, assay of *i*PHA depolymerase is difficult, because of the nature of the polymeric substrate where when isolated *in vitro*, PHA are amorphous and covered by a surface layer consisting of proteins (mainly phasins) (see above). Thus, the activity of *i*PHB depolymerases is assayed by using artificial detergent-coated (cholate, oleate) PHB granules as a substrate prepared from purified crystalline PHB (Jendrossek, 2009). The preparation of substrate for *in vitro* intracellular depolymerization of PHA by *i*PHA depolymerase has to distinguish between two types of substrates i.e. *n*PHA and artificial PHA granule (*a*PHA).

2.3.2.1 Native PHA granule

Several methods have been developed for preparation of *n*PHA granule and basically the method included several main steps; 1) crude extract of bacterial cell containing PHA is prepared through French press or sonication disrupted, 2) *n*PHB granules with intact surface layers are prepared from crude extracts by subsequent specific buffered glycerol or sucrose density gradient centrifugation steps, 3) isolated *n*PHA granules are stored at -20 ºC and dialyzed to remove glycerol when desired, 4) the protein-free PHB granules obtained by pretreatment with specific substances to oxidize and solubilize any organic material (Handrick et al., 2004a; Gebauer & Jendrossek, 2006; Chen et al., 2009; Sznajder & Jendrossek, 2011). Almost all *i*PHA depolymerase activities were reported to show strong dependence on the pretreatment of *n*PHB granules with trypsin, protease or an activator-like proteinaceous compounds to remove surface proteins, with the release of 3HB dimers and oligomers as the main hydrolytic products. Without the pretreatment, they show very little or no activity toward *n*PHB granules (Gao et al., 2001; Handrick et al., 2004a; Tokiwa & Calabia, 2004; Chen et al., 2009; Jendrossek, 2009). Only several *i*PHA depolymerase could efficiently hydrolyze *n*PHB

granules *in vitro* without pretreatment or activation by trypsin or activator-like compounds (Jendrossek, 2009)**.** Table 2.2 shows the different types of bacteria according to their particular types of PHA produced, depolymerization conditions and depolymerization efficiencies for *in vitro* depolymerization of intracellular PHA.

The effective protocol for storing polymer granules have been established which permits investigation of granule depolymerization and depolymerase activity. Previous studies clearly demonstrated that rapid shell freezing and storage at –70 °C enable the polymer granules to be maintained for a long time in distilled water without adversely affecting granule integrity or enzyme activity. Nonetheless, upon thawing and storage at 4 – 6 °C, accurate, quantitative determinations of depolymerase activity and granule depolymerization are possible only within the first ten days (Foster et al., 1996). In addition, the isolation of *n*PHB granules in glycerol density gradients also has the advantage that the granules can be stored under very low temperature (–20 °C) and stable without affecting the native state of the granules for a long period (Jendrossek, 2007). A major issue usually encountered during the preparation of *n*PHA granules substrates for the *in vitro* depolymerization studies is on how to prevent and stabilize the isolated amorphous granular from crystallizing. It is well known that the *n*PHA inclusions bodies are completely amorphous and extremely sensitive to chemical or physical stresses (Yoon & Choi, 1999; Jendrossek, 2007). An artificial PHA granule (*a*PHA) with similar characteristics with amorphous *n*PHA was developed to circumvent the aforementioned difficulties.

2.3.2.2 Artificial PHA granule

*a*PHB represents another form of amorphous PHB as a substrates for *in vitro* depolymerization of intracellular PHA. *a*PHB can be prepared from semicrystalline denatured PHB (*d*PHB) extracted from bacterial cell by dissolving *d*PHB in specific solvent (trichloromethane or acetone) and then emulsify the solution with an aqueous preparation of a surfactant (sodium dodecyl sulfate, sodium deoxycholate, oleate or cetyltrimethylammonium bromide) by stirring or ultrasonic treatment (Horowitz & Sanders, 1994; Handrick et al., 2004a; Gebauer & Jendrossek, 2006; Chen et al., 2009). A suspension of amorphous *a*PHB granules is obtained after the solvent evaporation. The amorphous state of *a*PHB granules is stable for a long time, if aggregation of the granules and close packing (e.g., by centrifugation) are avoided. *a*PHB could be used for *in vitro* analysis of PHA depolymerase activity or as substrate for isolation of PHA-depolymerizing bacteria (Jendrossek, 2007). Horowitz and Sanders (1994) reported that the failure of *n*PHB to crystallize is simply because of slow nucleation kinetics that are operative for small particles. Pure crystalline PHB can be reconstituted into submicron-size artificial granules when synthetic surfactants substitute the native granule coating, and can be recrystallized back when surfactants coating is removed by dialysis (Horowitz & Sanders, 1994). However, *a*PHB is not a physiological substance. All *in vivo* surface attached proteins are absent in *a*PHB. Therefore, it is difficult to predict whether an enzyme with PHA depolymerase activity *in vitro* using *a*PHB as substrate also has accessibility and degradability similar with *n*PHB granules *in vivo*. Therefore, the use of carefully isolated *n*PHB granules as a substrate for *in vitro i*-PHB depolymerase assay might lead to more meaningful results (Jendrossek & Handrick, 2002; Jendrossek, 2009).

Table 2.2: *In vitro* depolymerization of intracellular PHA by various bacteria.

Microorganisms	Substrate	Type of enzyme	Depolymerization conditions	Main hydrolytic product/ efficiency	References
Bacillus megaterium	*n*PHB without pretreatment, *a*PHB	Purified *i*PHA depolymerase	100 mM Tris-HCl, pH 8, 37 ˚C	3HB monomer	(Chen et al., 2009)
Bacillus thuringiensis	trypsin-treated *n*PHB granules, *a*PHB	Purified *i*PHA depolymerase	100 mM Tris-HCl, pH 8.0, 37 ˚C	3HB monomer	(Tseng et al., 2006)
Wautersia eutropha H16	*a*PHB	Purified *i*PHA depolymerase	100 mM Tris-HCl, pH 8.5, 30 ˚C	3HB oligomers	(Abe et al., 2005)
Rhodospirillum rubrum	trypsin-treated *n*PHB or activator compound present in cells	Purified *i*PHA depolymerase	100 mM Tris-HCl, pH 9, 1 mM $MgCl_2$	Monomer, dimer, trimer	(Handrick et al., 2004a; Handrick et al., 2004b)
Paracoccus denitrifican	protease-treated *n*PHB granules	Purified *i*PHA depolymerase	50 mM Tris-HCl, pH 9, 30 ˚C	3HB dimers and oligomers	(Gao et al., 2001)

Table 2.2: continued.

Microorganisms	Substrate	Type of enzyme	Depolymerization conditions	Main hydrolytic product/ efficiency	References
Ralstonia eutropha H16	*a*PHB	Purified *i*PHA depolymerase	40 µmol Tris-HCl, pH 9.0, 30 °C	3HB oligomers	(Saegusa et al., 2001)
Paucimonas lemoignei	*n*P(3HB) or *n*P(3HV), *a*PHB or *a*PHV	Purified *i*PHA depolymerase	100 mM Tris-HCl, pH 9, 1 mM $CaCl_2$, 40 °C	- Pentamer of 3HB or 3HV - no activity for *a*PHA	(Handrick et al., 2001)
Pseudomonas oleovorans	SDS-treated *n*PHA (PHN, PHPV, PHN+PHPV)	*i*PHA depolymerase in inclusion body isolation PHO, PHN, PHPV, PHN+PHPV	250 mM glycine-NaOH, pH 9, 30 °C, $MgCl_2$	- PHN > PHPV > PHN+PHPV - (PHPV + *i*-PHA depolymerase PHO) > (PHPV + *i*-PHA depolymerase PHPV)	(Foster et al., 1999)
Pseudomonas oleovorans	*n*PHO	*i*PHA depolymerase in inclusion body isolation PHO & solubilized from the granule	625 µmol of Tris-HC1 or Glycine-NaOH, pH 9, 187.5 µmol of MgCl2, pH 9, at 30°C.	(PHO + *i*-PHA depolymerase solubilized from the granule) > (PHO + *i*-PHA depolymerase in inclusion body isolation)	(Foster et al., 1996)

2.4 Extracellular Depolymerization of PHA

Extracellular depolymerization of PHA occurs after the polymer is being released from the cell (e.g., after cell lysis, death or by solvent extraction) or after the surface layer is damaged, the polymer denatures and becomes more or less crystalline (paracrystalline) (Handrick et al., 2004a; Gebauer & Jendrossek, 2006). In bacterial cells, *n*PHA are in the native, amorphous "rubbery" state, consists of highly mobile chains of carbon back bone in disordered conformation and are covered by a surface layer of mainly proteins. Upon extraction from the cell, after cell lysis or death, the surface layer of *n*PHA granules consists of proteins is rapidly damaged or lost and the polyester chains tend to adopt an ordered helical conformation and to develop a crystalline phase. This polymer is referred as denatured (crystalline) PHA (*d*PHA) (Handrick et al., 2004a; Jendrossek, 2007; Uchino et al., 2007; Chen et al., 2009; Bresan et al., 2016). *d*PHB, for instance is a partially crystalline polymer (typical degree of crystallinity 50–60 %) with an amorphous fraction characterized by the same glass transition temperature as native PHB (T_g ~ 0 °C) and a crystalline phase that melts in the range of 170–180 °C (Handrick et al., 2001; Handrick et al., 2004a; Tokiwa & Calabia, 2004; Jendrossek, 2007).

*d*PHA can be prepared by extraction and digestion of PHA from accumulating bacteria through solvent extraction (acetone, chloroform, dichloromethane) and chemical digestion (sodium hypochlorite) (Jendrossek, 2007; Anis et al., 2012; Anis et al., 2013). Crystalline scl-PHA, such as PHB and P(3HB-*co*-3HV) copolymers are also being produced by several companies so they are commercially available (Jendrossek, 2007). In extracellular depolymerization studies, *d*PHA acts as a substrate and exists in several states such as emulsified PHA, crude or precipitated PHA, solvent-cast PHA films, commercial PHA pellets, and others (Yu et al., 2005; Boyandin et al., 2013). The physicochemical properties of *d*PHA such as crystallinity, monomer or blending composition, lamellar thickness, molecular conformation and surface area contribute to

the efficiency of extracellular *d*PHA depolymerization as well as controlling the PHA degradability (Philip et al., 2007; Salim et al., 2012; Iwata, 2015). Studies about extracellular depolymerization of PHA are wider, extensive and very well understood as compared to intracellular depolymerization of PHA. Several studies on the extracellular *d*PHA depolymerization using enzymatic, thermal and chemical digestion methods have been reported (Yu et al., 2005; Rodríguez-Contreras et al., 2012).

2.4.1 Enzymatic hydrolysis

The ester bonds of PHA are the Archilles' heel of the polymer that can be hydrolyzed by a large variety of hydrolytic enzymes such as extracellular PHA depolymerase enzyme (*e*PHA depolymerase), esterase and lipase (Hermawan & Jendrossek, 2010; Yan et al., 2012; Ch'ng & Sudesh, 2013). These enzymes are categorized as serine hydrolases containing a catalytic triad (*Ser, Glu/Aps, His*) in their active sides. However, *e*PHA depolymerase contained a PHA binding domain which is different from the others (Yan et al., 2012).

Extracellular depolymerization of *d*PHA naturally is a utilization of exogenous carbon and energy source by a not-necessarily-accumulating microorganism. The source of this *d*PHA is PHA that are being released by those accumulating cells that have ceased to divide and undergo cell lysis. The ability to depolymerize PHA is widely distributed among bacteria and depended on the secretion of specific *e*PHA depolymerases that are carboxyesterases and capable to hydrolyse water-insoluble polymer into water-soluble monomers or oligomers (Handrick et al., 2001; Kim et al., 2007). Extracellular depolymerization of PHA by *e*PHA depolymerase is usually a heterogeneous process. Microorganisms are unable to transport the polymeric material directly into the cells where most biochemical processes take place because lack of water-solubility property and the size of the polymer molecules.

Therefore, the microorganisms will first excrete *e*PHA depolymerase which depolymerize the polymers outside the cells by adsorption of the enzyme on the biopolymer surface. This reaction of *e*PHA depolymerase enzymes must be in close proximity to its substrates binding domain of the enzyme for specific period of time for efficient activity. The second step is hydrolysis of the polymer chains by the active site (catalytic domain) of the enzyme into water-soluble products. As a consequence, if the polymer's molar mass can be sufficiently reduced to generate water-soluble intermediates, these can be transported into the microorganisms and fed into the appropriate metabolic pathways as nutrients within cells for biomass growth (Abe et al., 1998; He et al., 2001; Li et al., 2003; Tokiwa & Calabia, 2004; Rizzarelli & Carroccio, 2014; Shah et al., 2014,).

To date, almost 80 extracellular scl-*e*PHA depolymerases have been purified and characterized in terms of their biochemical and molecular biological properties from different microorganisms but relatively uncommon for mcl-*e*PHA depolymerase (Yoon & Choi, 1999; Kim et al., 2002; Kim et al., 2003; Kim & Rhee, 2003; Kim et al., 2007; Martínez et al., 2015). The characterized P(3HB) depolymerases have some common properties such as molecular weight (M_w) lower than 70 kDa, good stability at a wide range of pH (7.5 – 9.5), ionic strength (I) and temperature (Jendrossek & Handrick, 2002; Tokiwa & Calabia, 2004; Amara & Moawad, 2011; Sun et al., 2014). Usually, *e*PHA depolymerase works by partially depolymerizing crystallized *d*PHA or requires the presence of a *d*PHA crystalline phase because they do not bind well with the mobile amorphous phase to induce hydrolysis (He et al., 2001; Li et al., 2003; Arroyo et al., 2011). However, some *e*PHA depolymerase have broad specificity and able to utilize large variety types of polymers not limited to *d*PHA substrates alone (Jendrossek & Handrick, 2002; Tokiwa & Calabia, 2004). The heterogeneous enzymatic depolymerization mechanism of PHA has been developed by using folded chain single

crystals as model substrates, where enzymes are being 'shaved off' the molecular chains parallel to the chain folding plane at the edge of a lath-shaped PHB crystal. The chain scission proceeds by both *endo* and *exo* action to solubilize the edge material. The overall effect is called "processive degradation" which applied to highly ordered single crystals. As a result, the residual PHB retains the same molecular weight as before depolymerization (Yu & Marchessault, 2000). It was also reported that not all *e*PHA depolymerases have both *endo*- and *exo*-hydrolyse activities. The type of activity can be deduced from the end products whether they are only monomers, or monomers and dimers, or mixture of oligomers (Jendrossek & Handrick, 2002). Studies on enzymatic depolymerization of extracellular *d*PHA can be divided into three *viz*. in environment, *in vivo* and *in vitro*.

2.4.1.1 Environmental depolymerization of PHA

One of the unique features of PHA is their complete biodegradability in various environmental conditions caused mainly by microbial activity (Vigneswari et al., 2012). Various microorganisms which are PHA degraders in the natural environment will excrete specific *e*PHA depolymerase to hydrolyze *d*PHA within the vicinity of the cells and absorbed the resultant monomers and oligomers from decomposed compound to be utilized as a nutrients (carbon/energy source). These mechanisms play an important role in the metabolism of PHA and are ubiquitous in the environment (Doi, 1990; Thakor et al., 2005; Shah et al., 2008; Hiraishi & Taguchi, 2009). Studies on environmental depolymerization of *d*PHA usually are carried out in natural ecosystem such as in soil, activated sludge, river, compost, lake water and seawater (Doi, 1990; García-hidalgo et al., 2012; Boyandin et al., 2013). The degradability of *n*PHA not only depending on the physicochemical properties of *d*PHA but also on various biotic and abiotic factors such as microbial activity, pH, temperature, moisture, oxygen availability and salinity (Lee &

Choi, 1999; Ojumu et al., 2004; Tokiwa & Calabia, 2004; Arcos-Hernandez et al., 2012; Boyandin et al., 2013; Iwata, 2015).

The plate count and clear-zone methods are very efficient tools for evaluation of the population of polymer-depolymerizing microorganisms in the environment. The clear-zone method is widely used for the isolation and screening of polymer-depolymerizing microorganisms. The formation of clear zones around the colonies is an indication that the polymer is being hydrolyzed into water-soluble products by the relevant enzyme(s). The main advantage of the test is that it is generally fast and simple, and allows for simultaneous execution of a number of tests (Tokiwa & Calabia, 2004). Another significance of environmental depolymerization of extracellular *d*PHA is to proof that neat PHA can be degraded upon disposal of end usage (Salim et al., 2012). Usually, *d*PHA in the form of compact pellet (precipitated PHA) or PHA film with standardized weight and measurement are used in the studies. The polymers are buried in selected natural ecosystem. Investigations are performed within certain period of time with sampling at certain time intervals (Boyandin et al., 2013). The PHA-depolymerizing microorganisms can also be isolated from the vicinity where the polymers are buried.

Different types of *d*PHA are used as a substrate in a specific medium for isolating novel PHA-depolymerizing bacteria that produce specific *e*PHA depolymerase (Roy & Visakh, 2015). Serial dilutions of homogenized sample of microbial culture in a specialized medium are spread on mineral agar plates containing *d*PHA as a sole carbon source. The conventional clear-zone technique is applied for isolating the pure culture colony with high depolymerase activity among the microorganisms grown on the same medium (Kim et al., 2002; Gangoiti et al., 2012). The clear halos surrounding the microbial growth indicate the depolymerization of PHA as a substrate help to reveal a particular strain that secreted *e*PHA depolymerase (Jendrossek et al., 1996; Jendrossek, 2002; Yan et al., 2012; García-Hidalgo et al., 2013). A number of aerobic and anaerobic

microorganisms i.e. bacteria and fungi with the capacity to excrete *e*PHA depolymerase for depolymerizing *d*PHA have been successfully isolated from various environments such as *Acidovorax faecilis*, *Aspergillus fumigatus*, *Alcaligenes faecalis*, *Comamonas testosterone*, *Pseudomonas stutzeri*, *Streptomyces ascomycinicus* and others (Kim et al., 2007; Shah et al., 2008; García-Hidalgo et al., 2013; Ghosh et al., 2013).

2.4.1.2 *In vivo* depolymerization of PHA

The use of *d*PHA in *in vivo* applications as biodegradable material for sutures, microcapsules, bone plates, scaffolds and gauzes require serious attention in the study of *in vivo* depolymerization of extracellular PHA (Anderson & Dawes, 1990; Chen, 2010). The unique properties of these PHA are their biodegradability and excellent biocompatibility, because the rate of depolymerization of the material should equal to the regenerative rate of tissue, which makes them attractive and effectively utilized as scaffold in tissue engineering applications (Philip et al., 2007; Chen, 2010; Ansari & Amirul, 2013). In addition, the main advantage is a biodegradable material such as PHA can be inserted into human body without the need for subsequent removal. PHA has an ideal biocompatibility as it is a product of cell metabolism. 3-hydroxybutyric acid i.e. the product of depolymerization is normally present in blood. *In vivo* PHA are also depolymerized by the enzymes present in blood and animal tissues, which are particularly useful in the slow release of drugs and hormones (Philip et al., 2007; Verlinden et al., 2007). Meanwhile, the hydrolysis of P(3HB-*co*-4HB) yields 4-hydroxybutyrate (4HB), which is a natural human metabolite present in the brain, heart, lung, liver, kidney, and muscle (Vigneswari et al., 2012).

PHA such as P(3HB), P(3HB-*co*-3HV), P(4HB), P(3HB-*co*-4HB), P(3HB-*co*-3HHx) and P(3HHx-*co*-3HO) have been tested in animals for their *in vivo* physiological functions. These tests revealed that all these polymers are biocompatible in various host

systems. Evidence for PHB biodegradability in mice was obtained from observing the weight losses of PHB tablets (Anderson & Dawes, 1990; Chen & Wu, 2005). Hence, verified extraction procedures employed for PHA to be used in medical applications are needed to ensure highly pure end products, free of bacterial endotoxins, absence of halogenated solvents, non-degradation of the polymer and high efficacy (Philip et al., 2007; Verlinden et al., 2007).

2.4.1.3 *In vitro* depolymerization of PHA

In vitro enzymatic depolymerization of extracellular *d*PHA studies are useful to predict the depolymerization rate, evaluate the properties of the polymer and estimate the enzymatic or hydrolytic mechanisms. In recent years, PHA and their copolymers are widely used in the biomedical devices and biomaterial applications (Ansari & Amirul, 2013). Studies on polymer surface modification have been carried out by partial enzymatic depolymerization of polymer to create porous scaffold for tissue engineering. PHA are predominantly hydrophobic, and surface modification of the PHA is necessary to improve their hydrophilicity, biocompatibility, wettability, and surface charge to provide sufficient affinity for cell adhesion in order to its growth, hence suitable for biomedical applications (Guzmán et al., 2011; Ansari & Amirul, 2013).

As reported by Ansari et al. (2011), PHA depolymerases are favored to hydrolyze the P(3HB) chains on the surface of the P(3HB-*co*-4HB) solvent-cast films, thus increasing the 4HB molar fraction. Surface modification of PHA with high molar fraction of 4HB using enzymatic technique has been found to create highly porous scaffold with a large surface area-to-volume ratio, which makes them attractive as potential tissue scaffold in biomedical field (Martin & Williams, 2003; Ansari et al., 2011; Ansari & Amirul, 2013). The tissue scaffold must be designed to satisfy several requirements and mimicking the properties of living systems. The P(3HB-*co*-4HB) scaffolds have been

investigated as medical patches for controlled drug release or *in vivo* tissue regeneration (Vigneswari et al., 2012).

The *in vitro* depolymerization of extracellular *d*PHA studies carried out usually aimed to investigate the stability of the polymers in model systems that mimic *in vivo* conditions by varying values of several factors that possibly affect enzymatic hydrolysis of *d*PHA including substrate size, PHA depolymerase concentration, incubation time, pH, medium salinity and temperature. Investigations using solvent-cast films have also shown that the rate of enzymatic depolymerization is affected by the chemical structure of individual co-monomer units, monomer proportions, different morphology, molecular mass and the crystallinity of samples. The polymer depolymerization is monitored by tracking the reduction in molecular mass and degree of crystallinity (Philip et al., 2007; Ansari & Amirul, 2013).

Furthermore, the production of (*R*)-3HB is also made possible *via* enzymatic depolymerization of extracellular *d*PHA. For instance, the use of thermophilic *Streptomyces* sp. MG with strong hydrolytic activity for depolymerization of PHB into (*R*)-3HB monomer. An added advantage with this strain is its stability at elevated temperature (50 ºC), which would minimize contamination problems not only during fermentation, but also during enzymatic depolymerization of PHB. Furthermore, (*R*)-3HB recovery is relatively easy since the cells aggregate to form clumps after the fermentation process. Production of (*R*)-3HB from PHB by hydrolytic enzyme is known to be economically feasible in terms of (*R*)-3HB recovery. Consequently, (*R*)-3HB can be recovered from plastic wastes that contain PHB (Tokiwa & Ugwu, 2007).

2.4.2 Thermal hydrolysis of PHA

Studies on the thermal depolymerization of *d*PHA have typically been carried out using thermogravimetric (TGA) and pyrolysis methods (Daly et al., 2005).

Thermodegradation of *d*PHA would have a dramatic effect on its thermal and physical properties, and can results in a progressive reduction in molecular weight concomitantly generating useful hydroxyl acid oligomers with favorable end groups for various platform applications (Aoyagi et al., 2002; Sin et al., 2011a; Sin et al., 2011b; Kwiecień et al., 2014; Sin et al., 2014). Thermal decomposition takes place in absence of organic solvents and other chemicals hence justifies the method to be producing low molecular weight PHA as green chemistry (Sin et al., 2011b). Moreover, understanding of thermal stability and thermal depolymerization behavior has its own importance to achieve practical manufacturing *modus operandi*. They must be sufficiently stable to undergo processing at elevated temperatures for downstream stages, application, and thermal recycling (Aoyagi et al., 2002; Daly et al., 2005; Sin et al., 2010).

At moderate temperatures, thermodegradation of scl-*d*PHA occurs almost exclusively *via* a random chain scission mechanism involving a six-member ring transition state or β-hydrogen-elimination reaction. This mechanism gives almost exclusively unsaturated ends for the products such as unsaturated carboxyl acids and unsaturated esters (Yu & Marchessault, 2000; Aoyagi et al., 2002; Nguyen et al., 2002; Fraga et al., 2005; Sin et al., 2010; Rodríguez-Contreras et al., 2012). In contrast, mcl-*d*PHA composed of six to fourteen carbon monomers, with aliphatic *R* side chain are having 3, 5, 7, 9 or 11 carbons. These bulky *R* side chains could hinder the formation of a stable 6-membered ring ester intermediate. Thus, the thermal depolymerization mechanism is hypothesized to involve α-chain scission of the mcl-PHA polymer chains *via* hydrolytic ester bond cleavage. The main depolymerization products are a mixture of low molecular weight oligomeric hydroxyalkanoic acids from the hydrolysis of the ester linkages. At higher temperature, a proportion of the depolymerization products could undergo dehydration of hydroxyl end groups, giving rise to alkenoic acid as secondary products (Sin et al., 2010; Sin et al., 2011a).

2.4.3 Chemical hydrolysis of PHA

The PHA produced from bacteria have a relatively high molecular mass (M_w) around 200 – 3000 kDa. For such applications, more manageable molecular masses, around 1-5 kDa are required for optically active specialty polymers synthesis such as molecular design in block and graft copolymers. Therefore, chemical hydrolysis employing strong acid and base is frequently used to depolymerize the natural *d*PHA to low molecular weight products. The presence of double bonds at the chain ends can be used advantageously in a variety of reaction schemes (Yu & Marchessault, 2000; Ravenelle & Marchessault, 2002; Rodríguez-Contreras et al., 2012). Hydrolysis products of *d*PHA after depolymerization under acidic or alkaline conditions are usually detected in the form of crotonic acid or as (*R*)-3HB acid (Tokiwa & Ugwu, 2007). Heterogenous hydrolysis mechanism by chemical route shows that the scission of PHB chains occur at the surfaces and interfaces of the lamellar crystals. The unsaturated end group is formed from dehydration of the chain ends by *β*-elimination after ester hydrolysis, which is different from the mechanistic steps in the formation of unsaturated ends in thermal decomposition of PHB (Yu & Marchessault, 2000).

de Roo et al. (2002) developed a novel and efficient method for the production of enantiomerically pure 3HA and 3HA methyl esters. The *d*PHA is isolated *via* solvent extraction and later hydrolyzed by acid methanolysis. The 3HA methyl ester mixture obtained is recovered by distillation process into several fractions. Subsequent saponification of the purified methylester fractions yields the corresponding 3HA. A wide range of functionalized enantiomerically (*R*)-3HA synthons can be produced by methanolysis of *d*PHA containing predefined chemical groups, and more importantly conducted under mild conditions (de Roo et al., 2002; Chen & Wu, 2005).

2.5 Analysis of PHA Depolymerization

In PHA depolymerization studies, there are many types of analyses available to determine the depolymerization rate of PHA, hydrolytic enzyme activities, products of depolymerization and the characteristics of depolymerized PHA. These analyses are important to assess the properties and yield information on the behavior of PHA before and after depolymerization. The typical analysis involved are summarized in Table 2.3.

Table 2.3: Typical analyses employed in PHA depolymerization studies.

Analysis	Purpose	References
Depolymerization rate and enzyme activity determination		
Weight loss measurements	Practicable when the depolymerization of macroscopically visible specimens of polymer samples (e.g. PHB films) is determined over a time scale of many hours	(Stuart, 1996; Gebauer & Jendrossek, 2006)
Clearing zone assay	Formation of clear hydrolytic zone on agar plates containing PHA as substrates revealed strain secreted enzyme. Different diameter of clear zone show variable degree of enzyme activity	(Jendrossek, 2007; Yan et al., 2012)
Photospectroscopy or spectrophotometer	Turbidimetric assay of opaque suspensions of PHA, the diameter of PHA decrease upon hydrolysis resulting in a decrease of the optical density of the PHA suspension	(Jendrossek, 2007; Chen et al., 2009)
pH-stat assay	Depolymerase activity assay by titration of the released acid based on online determination of NaOH consumption rates necessary to neutralize 3HB acid and/or 3HB oligomers produced during the hydrolysis reaction. High sensitivity and accuracy method. Not provide any information on the type of products (monomer versus oligomers)	(Handrick et al., 2004a; Gebauer & Jendrossek, 2006)
GC (Gas Chromatography)	To determine intracellular PHA depolymerization rate, and widely used method for quantitative analysis the polymer composition. Provide all type information of monomers produce	(Foster et al., 1999; Tokiwa & Ugwu, 2007).

Table 2.3: continued.

Analysis	Purpose	References
Characteristics of PHA before and after depolymerization		
GPC (Gel Permeation Chromatography)	Determination in changes of molecular weight and molecular weight distribution	(Yu & Marchessault, 2000; Sin et al., 2011b; Kanmani et al., 2016)
FTIR (Fourier Transform Infrared Spectroscopy)	Changes in molecular vibrations could be noticed in the FTIR spectra	(Kanmani et al., 2016)
DSC (Differential Scanning Calorimetry)	Determination of the melting temperature of the polymer	(Kanmani et al., 2016).
TGA (Thermogravimetric analysis)	A technique in which the change in mass or total weight loss of a polymer sample is measured while the sample is heated at a constant rate either in an inert or oxidizing atmosphere	(Daly et al., 2005; Kanmani et al., 2016)
^{1}H, ^{13}C and ^{31}P NMR (Nuclear Magnetic Resonance)	Determination of the unsaturated contents, characterize the end groups, chemical shift, molecular weights and the changes in molecular structure of the polymer	(Yu & Marchessault, 2000; Kanmani et al., 2016)

Table 2.3: continued.

Analysis	Purpose	References
MALDI (Matrix Assisted Laser Desorption Ionization)	Direct measurement of the molecular weights and molecular weight distributions	(Yu & Marchessault, 2000)
(SEM) Scanning electron micrographs	Revealed the alterations in polymer surface morphology such as roughening, grooves, cavities and disintegration of the films	(Shah et al., 2008; Kanmani et al., 2016).
Determination and isolation of depolymerization product		
HPLC (High Pressure Liquid Chromatography)	Allow the separation, purification and quantification of polymer hydrolysis product	(Gebauer & Jendrossek, 2006)
GCMS (Gas Chromatography Mass Spectrometry)	Identification of polymer hydrolysis product	(Allen et al., 2012)

2.6 Applications of (*R*)-3HA

To date, more than 150 different types of carboxylic acids in the PHA family have been identified (Foster et al., 1999; Lee et al., 1999; Handrick et al., 2004a; Ren et al., 2005). The hydrolysis of PHA can produce enantiomerically pure (*R*)-3HA, which can be used as a chiral starting material for many industrial and medical applications. (Chen et al., 2009). Several possible PHA depolymerizations methods for the production of (*R*)-3HA and its co-polyesters have been investigated (Ren et al., 2005; Ruth et al., 2007; Allen et al., 2012; Kawata et al., 2012). In enzymatic hydrolysis, the main reaction products identified from *in vitro* extracellular PHA depolymerization systems of *Pseudomonas fluorescens* GK13 (*PhaZ*GK13) are dimers, whereas in the case of *in vivo* intracellular polymer depolymerization by the *PhaZ* depolymerase of *Pseudomonas putida* KT2442, the main reaction products are monomers (Martínez et al., 2014). The production of low molecular weight of PHA, oligomers, dimers or monomers (*R*)-3HA by chemical and thermal depolymerization also have been reported (Sin et al., 2011b).

The (*R*)-3HA contains a chiral center and two functional groups; hydroxyl (-OH) and carboxylic acid (-COOH), which are relatively easy to be modified chemically. The new chiral centers also can be introduced into these molecules to synthesize new compounds (Lee et al., 1999; Ballistreri et al., 2001; Hölscher et al., 2010; Gao et al., 2011; Allen et al., 2012). (*R*)-3HA has been known to exhibit some antimicrobial, insecticidal, and antiviral activities. Therefore, (*R*)-3HB could function as chiral building blocks or optically active compounds for synthesis of fine chemicals; such as antibiotics, vitamins, flavors, aromatics, pheromones, and others (Lee et al., 1999; Ren et al., 2005; Ruth et al., 2007; Tokiwa & Ugwu, 2007; Chen et al., 2009).

(*R*)-3HA also have been reported to have antimicrobial properties. For example, (*R*)-3-hydroxy-*n*-phenylalkanoic acids can be used to effectively eliminate *Listeria monocytogenes*, which is a ubiquitous microorganism with the capability to multiply at

refrigeration temperatures, and resistant to both high temperatures and low pH values. The food pathogen has become an important concern in many countries during the past several decades (Ruth et al., 2007; Chen, 2009).

Furthermore, (*R*)-3HB has been used as a starting material to produce carbapenem, which in turn become a promising material in the production of *β*-lactam antibiotics and microlides. Optically pure monomers have also been used in the production of sex hormones and *S*-citronellol, a fragrance (Lee et al., 2000; Philip et al., 2007; Chen, 2009).

In addition, novel chiral polyesters are also being synthesized from (*R*)-3HA as building blocks of chiral compounds such as 3-hydroxy-3-cyclopro-pylpropionate and 3-hydroxy-4-chlorobutyrate, and their CoA thioester derivatives. The two new polymers are crystalline in nature and both possess one chiral center each. Dendrimers is a novel class of polymers which was also being synthesized using (*R*)-3HB for application as *in vivo* drug carriers. Novel *β*- and *γ*– peptides or amino acids have also been produced by replacing amino acids with 3-hydroxybutyrate residues in peptides and by replacing the chain-bound oxygens in 3HB or 4HB with N-H group. Amongst the useful characteristics, they are stable to peptidases attack, resistance to environmental microbial depolymerization and good stability in mammalian serum. Some of them also exhibit useful antibacterial, antiproliferative and haemolytic properties (Chen & Wu, 2005; Philip et al., 2007; Chen, 2009).

Chiral hydroxyalkanoates are also known to possess novel nutrition qualities. Studies have shown that (*R*)-3HB confers partial protection and stability to neurons during glucose deprivation. There are evidences showing that they could serve as energy substrates in the attempt to increase cardiac efficiency and thus, prevent brain damage (Tokiwa & Ugwu, 2007). Oligomers of PHA have been applied as nutrients for animals. For example, oligo-HA was used as a nutrient supplement in rat and human (Chen & Wu,

2005). Tasaki et al. (1999) used ketone bodies including 3HB dimer and trimer residue mixture as a novel nutrition source. When the mixture of 3HB oligomers was injected into rats, they were observed to be completely converted into monomers. Hence, 3HB has the potential to act as an energy substrate in injured patients since it has high penetration rates and rapid diffusion in peripheral tissues (Tasaki et al., 1999; Chen & Wu, 2005; Philip et al., 2007; Chen, 2010).

Different processes have been reported to obtain enantiopure 3-hydroxy esters or chiral (*R*)-3HB by both chemical synthesis and biotechnological processes (Kawata et al., 2012). Even though the production of optically active (*R*)-3HB by chemical synthesis is possible, the process is significantly difficult, very complex with multistep reactions and expensive because a large amount of organic solvents is required, and the production efficiency was rather low (Lee et al., 1999; Ren et al., 2005; Tokiwa & Ugwu, 2007; Allen et al., 2012; Gangoiti et al., 2012; Kawata et al., 2012). Thus, the production of various chiral (*R*)-3HAs from different types of PHA by innovative depolymerization techniques are convenient since they are expected to be environmentally clean process, cost effective, absence of organic solvent usage and more viable compared to other conventional methods (Lee et al., 2008; Kawata et al., 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

Pseudomonas putida Bet001 was used throughout this research. The culture was obtained from Bioprocess and Enzyme Technology Laboratory (BET Lab), Institute of Biological Science, Faculty of Sciences, University of Malaya. It was isolated from palm oil mill effluent (POME) (Gumel et al., 2012).

3.1.2 Media

Two types of media were used for biosynthesis of mcl-PHA by *P. putida* Bet001:

3.1.2.1 Nutrient rich medium

Nutrient rich (NR) medium is a complex medium primarily used to enhance the growth of the bacterium. The composition to prepare 1 L of NR medium is as follows:

Table 3.1: Nutrient rich medium.

Ingredients	Amount (g L^{-1})
Yeast extract (Bacto TM, France)	10.0
Nutrient broth (OXOID, England)	15.0
$(NH_4)_2SO_4$ (Sigma Aldrich, Germany)	5.0

The materials were weighed, dissolved and mixed in distilled water using a magnetic stirrer. The medium was sterilized using autoclave at 121 ºC, 103 kPa for 15 minutes.

3.1.2.2 Mineral salts medium

Mineral salts medium (MSM) is a defined medium with limiting nitrogen source concentration to induce mcl-PHA accumulation in the bacterial culture. The composition to prepare 1 L of MSM is as follows:

Table 3.2a: Mineral salts medium.

Ingredients	Amount (g L^{-1})
$NaNH_4HPO_4$ $4H_2O$	3.5
KH_2PO_4	3.7
K_2HPO_4	5.7
$MgSO_4$ $7H_2O$	1.0 % *v/v*
Trace elements	0.1 % *v/v*

The materials were weighed, dissolved and mixed in distilled water using a magnetic stirrer. The medium was sterilized using autoclave at 121 ºC, 103 kPa for 15 minutes. Separate sterile solutions of 0.1 M $MgSO_4$ $7H_2O$ (1.0 % *v/v*) and 0.1 % *v/v* trace elements (MT) solution were added aseptically to the sterile MSM liquid at room temperature (30 ± 2 °C). The trace elements component dissolved in 1 L of hydrochloric acid (HCl) 0.1 M consisted of:

Table 3.2b: Trace elements.

Ingredients	Amount (g L^{-1})
$CaCl_2$ $2H_2O$	1.47
$CoCl_2$ $6H_2O$	2.38
$CuCl_2$ $2H_2O$	0.17
$FeSO_4$ $7H_2O$	2.78
$MnCl_2$ $4H_2O$	1.98
$ZnSO_4$ $7H_2O$	0.29

3.1.2.3 Carbon substrates

There are several types of carbon substrates used in the MSM medium for accumulation of mcl-PHA by *P. putida* Bet001. Excess of carbon substrate with carbon-to-nitrogen (C/N) ratio at 20 mol:mol (except stated otherwise) was used in the medium:

Table 3.3: List of carbon substrates for biosynthesis of mcl-PHA.

Carbon sources	Carbon number
Dodecanoic acid/ Lauric acid (Merck, Germany)	$C_{12:0}$
Tetradecanoic acid/ Myristic acid (Merck, Germany)	$C_{14:0}$
Hexadecanoic acid/ Palmitic acid (Merck, Germany)	$C_{16:0}$
Octadecanoic acid/ Steric acid (Merck, Germany)	$C_{18:0}$
cis-9-Octadecenoic acid/ Oleic acid (Sigma Aldrich, Germany)	$C_{18:1}$
Saponified palm kernal oil (SPKO)	-

3.1.3 General instruments

3.1.3.1 Shaker-incubator

The bacterial cultures in rich medium, MSM, and for *in vivo* depolymerization of intracellular mcl-PHA in MSM were incubated on the orbital shaker-incubator (Daihan LabTech®, LSI-1, Korea). The temperature and agitation speed were fixed at 25 ºC and 200 rpm, respectively.

3.1.3.2 Incubator

For *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA in buffer solutions, the culture solutions were statically placed in an incubator (Hotech, 624, Taiwan) at 30 ºC (except stated otherwise).

3.1.3.3 Centrifuge

Centrifugation was carried out by using mini centrifuge (Thermo-Line, MLX, China) for small volume samples (1 mL) at 30 °C, 2800 × *g* and ultracentrifuge (Thermo Scientific, Sorval RC-5C Plus, USA) for large volume samples (10 to 30 mL) at 4 °C, 3578 × g for 5 minutes.

3.1.3.4 Sterilizer

All the materials such as medium and apparatus to be used under sterile condition were sterilized using high pressure steam sterilizer autoclave machine (Tomy, SX-500, Japan) at 121 ºC, 103 kPa pressure for 15 minutes. Heat-sensitive materials were sterilized using 0.22 µm filter-sterilization (Sartorious, Minisart, Germany). All aseptic works were carried out in a laminar air-flow cabinet equipped with biohazard protection (Gelman Sciences, BH120, Australia).

3.1.3.5 Water bath sonicator

Bacterial cell suspension was lysed using ultrasonic bath sonicator (Elmasonic P, 30H, Germany). The frequency, power output and temperature were fixed at 37 kHz, 30 % and < 25 ºC, respectively.

3.1.3.6 Drying oven

The bacterial biomass after biosynthesis of mcl-PHA and depolymerization process was dried in a heating drying oven (Constance, DHG9036A, Germany) at 70 ˚C until constant weight.

3.1.3.7 Freeze drier

The supernatant from depolymerization process was collected and acidified using concentrated HCl (pH 1) and was kept in a freezer at -20 ºC overnight. Then, the samples were freeze-dried in a vacuum freeze-drier machine (LaboGene, Coolsafe 110-4, Denmark) at -108 ºC until the entire samples were fully dried.

3.1.3.8 Rotary evaporator

Solvent evaporation and concentration was carried out using rotary vacuum evaporator (Yamato, RE 300, Japan) at 70 ºC.

3.1.3.9 Spectrophotometer

The optical density (OD) measurement at the required wavelength was performed using the spectrophotometer machine (Jasco, V-630 UV/VIS, Japan) equipped with temperature controller (Jasco, ECH-716, Japan).

3.1.3.10 pH meter

The pH value was determined using a digital pH meter (Eutech Instruments, Singapore). The pH meter was calibrated in the buffer solutions of pH 4 and 7, or pH 7 and 10.

3.1.3.11 Thermometric titrator

The determination of residual fatty acids and ammonium were carried out using a thermometric titrator (Metrohm, 859 Titrotherm® Switzerland).

3.1.4 Reagent preparation

3.1.4.1 Buffer solutions

There are several types of buffer solutions used in the depolymerization studies. The following are the recipes for selected buffer solutions used in this study:

i) Glycine-HCl

25 mL 0.2 M glycine and *x* mL 0.2 M HCl was combined and diluted to 100 mL with deionized water (DI). The pH of the buffer was adjusted to 2, 3, and 4 using appropriate amount of concentrated NaOH.

ii) Sodium acetate

The proportion of 0.1 *N* acetic acid and 0.1 *N* sodium acetate was combined while the pH of the buffer was adjusted to 4, 5, and 6 using appropriate amount of concentrated NaOH.

iii) Phosphate buffer

The appropriate volumes of stock solutions of 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 was mixed. The equal volume of DI was added while the pH was adjusted to 6, 7, and 8 using appropriate amount of concentrated HCl and NaOH.

iv) Tris-HCl

0.2 M Tris base was dissolved in DI and the pH was adjusted with an appropriate amount of concentrated HCl to pH 7, 8, and 9.

v) Glycine-NaOH

25 mL 0.2 M glycine stock was combined with *x* mL 0.2 M NaOH and diluted with DI to make 100 mL solution. pH was adjusted to 9, 10, and 11 using appropriate amount of concentrated NaOH

3.1.4.2 Media preparation for bacterial culture maintenance

i) Nutrient agar (NA)

23 g of NA powder (BD Difco TM, USA) containing beef extract, peptone and agar was dissolved in 1 L distilled H_2O.

ii) Nutrient broth (NB)

8 g of NB powder (Merck, USA) containing peptone and meat extract was dissolved in 1 L distilled H_2O.

iii) Glycerol 40 % *v/v*

40 mL of glycerol was mixed with 60 mL distilled water.

3.1.4.3 Solutions preparation

i) 0.9 % *w/v* saline solution

A stock solution of 0.9 % *w/v* saline solution was prepared by dissolving 9.0 g sodium chloride in distilled water until 1.0 L final volume.

ii) 0.1 M $MgSO_4.7H_2O$

2.46 g of $MgSO_4.7H_2O$ was dissolved in distilled water in 100 mL final volume.

iii) 0.1 M HCl

0.1 M HCl was prepared by adding 41.4 mL of 37 % HCl (Friendemenn Schmidt, Western Australia) into 1.0 L volumetric flask and made up to 1.0 L with distilled water.

iv) Preparation of SPKO

Saponification of palm kernel oil (SPKO) was prepared according to (Tan et al., 1997). 2.8 g of NaOH was dissolved into 100 mL of absolute ethanol. 8 g of palm kernel oil (PKO) was added and mixed well inside a round-bottom flask. The mixture was refluxed gently for 1 hour while swirled from time to time. Subsequently, the flask was cooled under running tap water. Excess ethanol was

evaporated by rotary vacuum evaporator leaving behind sodium salt of the fatty acids (SPKO). The SPKO was further dried and stored in room temperature.

3.1.4.4 Preparation of 3-hydroxyalkanoic methyl ester standards for gas chromatography (GC) analysis

Several GC standards were used to determine the monomer concentration of mcl-PHA and representative retention time for each monomer peaks. The retention time for each monomer standard was determined using GC (Table 3.4).

Table 3.4: 3-hydroxyalkanoic methyl ester standards with their retention times.

3-hydroxyalkanoic methyl ester standards	Retention time (minutes)
Methyl 3-hydroxyhexanoate (C_6) (Sigma Aldrich, Germany)	5.703 ± 0.319
Methyl 3-hydroxyoctanoate (C_8) (Larodan, Sweden)	8.512 ± 0.408
Methyl 3-hydroxydecanoate (C_{10}) (Larodan, Sweden)	11.102 ± 0.429
Methyl 3-hydroxydodecanoate (C_{12}) (Larodan, Sweden)	13.401 ± 0.419

All GC standards were prepared in 10 000 ppm for initial stock. Subsequently, the stocks were diluted in DCM (Merck, USA) by serial dilution to obtain 100, 200, 300, 400 and 500 ppm concentration for the construction of standard calibration plot. The relationship between peak area and individual 3-hydroxyalkanoic acid methyl ester standards as shown in Figure 3.1a – d below:

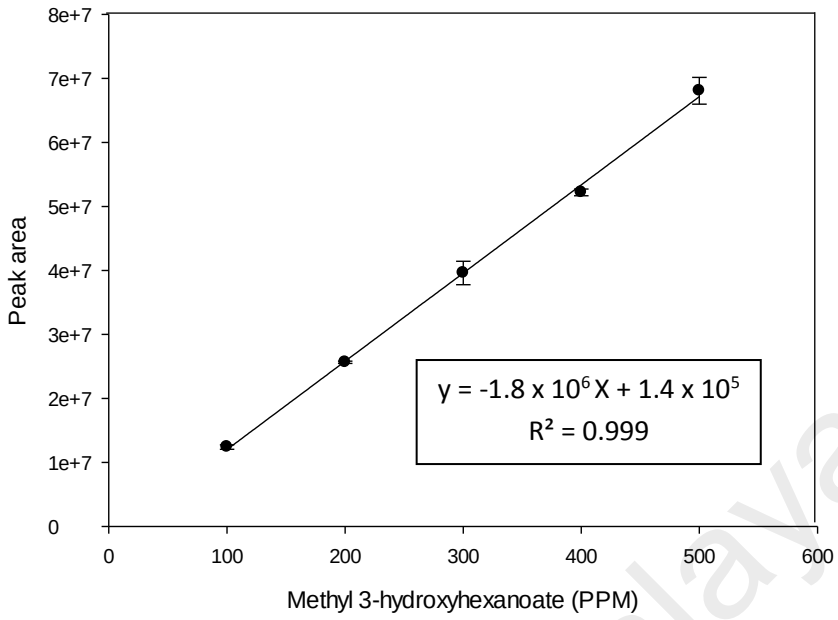


Figure 3.1a: Standard calibration of methyl 3-hydroxyhexanoate.

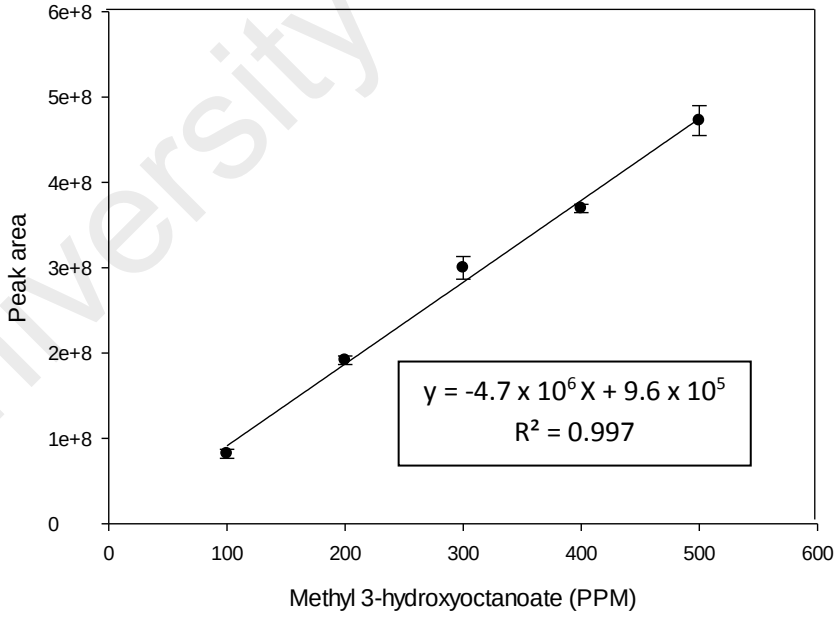


Figure 3.1b: Standard calibration of methyl 3-hydroxyoctanoate.

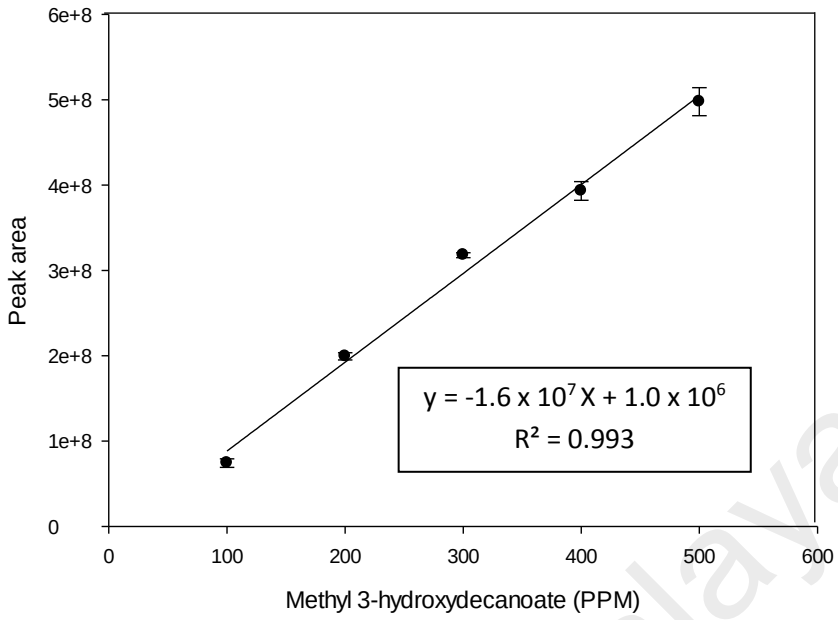


Figure 3.1c: Standard calibration of methyl 3-hydroxydecanoate.

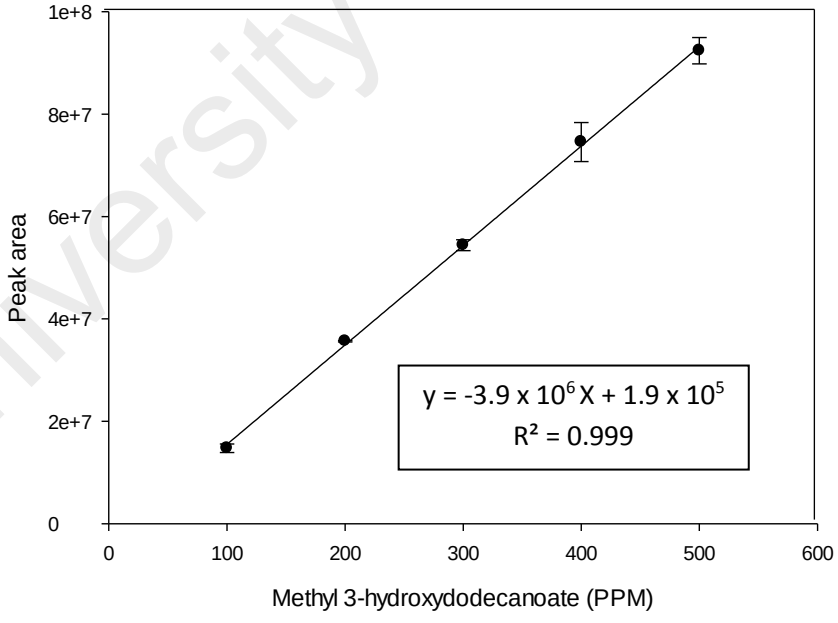


Figure 3.1d: Standard calibration of methyl 3-hydroxydodecanoate.

3.2 Methods

3.2.1 Strain maintenance

The bacterial strain was kept in 20 % *v/v* glycerol stock and stored at -20 ºC for 2 or 3 months. After being stored for 2 or 3 months, the bacterial stock was activated in nutrient broth (NB). Then, a loopful of this culture was streaked onto nutrients agar (NA) plates to obtain single colonies and to detect any possible contamination. The morphology of bacterium was observed under light microscope for visual inspection of the morphology. A selected single colony was transferred into fresh NB for new bacterial stock preparation. Bacterial culture suspension in NB was mixed with 40 % *v/v* glycerol in 1:1 *v/v* ratio. Several stocks were made to ensure the availability of the strain throughout the study and kept in -20 ºC.

3.2.2 Standard calibration of *P. putida* Bet001 biomass

A standard calibration of *P. putida* Bet001 biomass was prepared by culturing the bacterial cells in NR in 100 mL per each 250 mL shake-flasks at 25 ºC with 200 rpm agitation for 24 hours. The bacterial biomass was harvested and washed twice by centrifugation and re-suspend in 0.9 % *w/v* of saline solution. The bacterial suspension was divided into two:

i) 10 mL of bacterial suspension in each five empty aluminum boats were dried in a hot-air oven (70 °C) until constant weight. The dry cell weight (DCW) in g L^{-1} was determined by averaging the sample replicates;

ii) Serial dilution of the bacterial suspension in 0.9 % *w/v* of saline was carried out. OD reading of each dilution was taken at 600 nm using spectrophotometer.

The standard calibration was constructed by plotting OD values against the DCWs. The actual DCWs at different ODs were determined from average DCWs divided by dilution factor (DF). The standard calibration curve of *P. putida* Bet001 biomass is shown in Figure 3.2. The biomass concentration was calculated based on the relationship:

$$y = 2.6543x + 0.0048 \qquad \text{(eq. 1)}$$

where y is the OD reading at 600 nm and x is the calculated biomass weight (g L^{-1}). 1 unit OD_{600} is equivalent to 0.37 g L^{-1} of DCW.

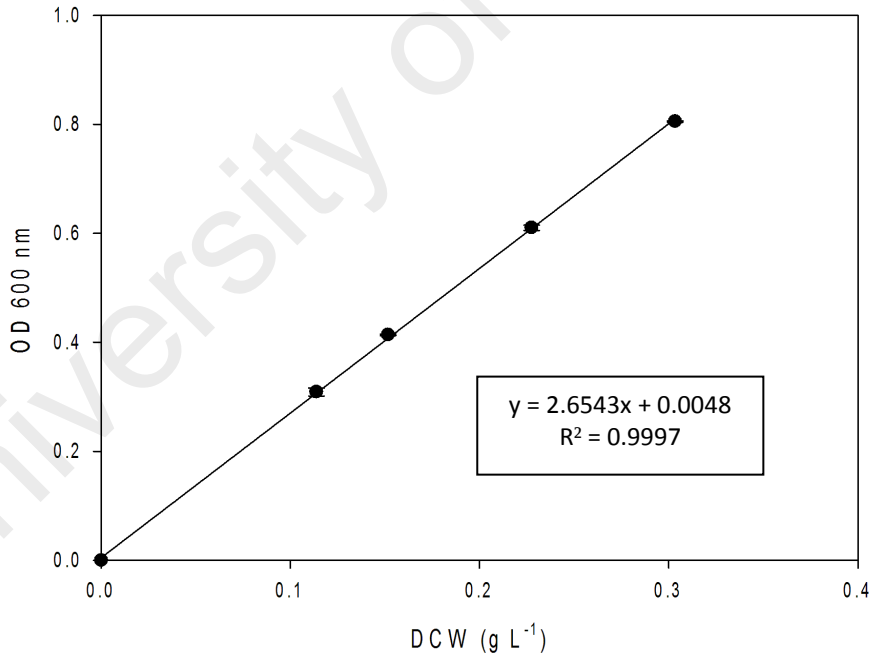


Figure 3.2: Standard calibration of *P. putida* Bet001 dried biomass.

3.2.3 Biomass estimation

Bacterial DCW was rapidly estimated by taking the optical density (OD) readings of the cell culture. The OD was determined at 600 nm of wavelength (OD_{600}) using spectrophotometer. The samples were diluted with distilled water if OD values exceeded 0.8. A standard calibration for *P. putida* Bet001 biomass against OD_{600} was prepared by serial dilution of known amount of cell biomass. 1 unit of OD_{600} is equivalent to 0.37 g L^{-1} of DCW. The biomass concentration was calculated by the following formula:

$$\text{DCW (g L}^{-1}) = OD_{600} \times dilution\ factor \times 0.37 \qquad \text{(eq. 2)}$$

3.2.4 Biosynthesis of mcl-PHA by *P. putida* Bet001

P. putida Bet001 isolated from POME was used in this study (Gumel et al., 2012). Bacterial culture from glycerol stock was re-constituted into NB for 24 hours prior to cultivation in NR medium. Bacterial cells solution (1 mL) from NB was aseptically inoculated into 100 mL NR per each 250 mL shake-flasks. The culture was incubated in a shaking incubator at 25 ˚C, 200 rpm agitation for 24 hours. Subsequently, the harvested culture broth was centrifuged at 4 ˚C, 3578 × *g* for 5 minutes and the recovered biomass was washed twice with 0.9 % *w/v* saline solution. The cell suspension in sterile distilled water (1 g L^{-1}) was inoculated aseptically into PHA production medium MSM (100 mL per each 250 mL shake-flasks). It was supplied with lauric acid (C_{12}) (except stated otherwise) as a sole carbon and energy substrate but with limited nitrogen source to obtain C/N ratio at 20 (mol:mol) (except stated otherwise). Following inoculation, the flasks were incubated in aerobic condition at 25 ˚C and 200 rpm for 48 hours (except stated otherwise). After cultivation, the harvested culture broth was centrifuged at 4 ˚C, 3578 × *g* for 5 minutes and the recovered biomass was washed twice with 0.9 % *w/v* saline solution and *n*-hexane (Merck, USA) to remove residual fatty acids. The bacterial mass

was dried in an oven at 70 ˚C until constant weight. The dried biomass was used to determine the mcl-PHA content and monomer composition using GC (Amirul et al., 2008). Selected process parameters *viz*. carbon source, C/N ratio, initial cell concentration, incubation time were studied to enhance the biomass and mcl-PHA biosynthesis. The residual carbon source concentration was determined at different incubation times.

3.2.4.1 Effects of various carbon substrates on cell dry weight and mcl-PHA content produced by *P. putida* Bet001

Different carbon substrates as listed in subsection 3.1.2.3 were used to study the effects of various carbon substrates on cell dry weight and mcl-PHA content produced by *P. putida* Bet001. The concentration of each carbon substrate used was fixed at C/N 20. The ensuing procedures were carried out as in section 3.2.4. The most productive carbon substrate was used for further experiments.

3.2.4.2 Effects of different C/N ratios on cell dry weight and mcl-PHA content produced by *P. putida* Bet001

Different C/N ratios of lauric acid 10, 15, 20, 25 and 30 were used to study the effects of various C/N ratios on cell dry weight and mcl-PHA content produced by *P. putida* Bet001. The ensuing procedures were carried out as stated in section 3.2.4. The most productive C/N ratio was used for further experiments.

3.2.4.3 Effects of different initial cell concentrations on cell dry weight and mcl-PHA content produced by *P. putida* Bet001

Different initial cell concentrations inoculated into mcl-PHA production medium ranging from 1 to 5 g L^{-1} were used to study their effects on cell dry weight and mcl-PHA

content produced by *P. putida* Bet001. The ensuing procedures were carried out as stated in section 3.2.4. The most suitable initial cell concentration was applied for further experiments.

3.2.4.4 Effects of different incubation times on cell dry weight and mcl-PHA content produced by *P. putida* Bet001

Different incubation times ranging from 12 to 72 hours were applied to study the effects of different incubation times on cell dry weight and mcl-PHA content produced by *P. putida* Bet001. The ensuing procedures were carried out as stated in section 3.2.4. The most suitable incubation time was employed for further experiments.

3.2.4.5 Determination of monomer composition in mcl-PHA synthesized from lauric acid as a sole carbon source

The monomer composition of mcl-PHA synthesized from lauric acid as a sole carbon source was determined when the optimal parameters for biosynthesis of mcl-PHA were applied. The ensuing procedures were carried out as stated in section 3.2.4.

3.2.4.6 Determination of residual lauric acid in the culture broth

The residual lauric acid was determined from the cell-free liquid culture using thermometric titrator. The method is based on the measurement of heat released from exothermic reaction between OH^- and H^+ species. For sample preparation, the residual lauric acid was separated using *n*-hexane from culture broth and diluted using 2-propanol. Samples were titrated with 0.1 M of standard potassium hydroxide (KOH) in isopropanol in the presence of *para*-formaldehyde as titration end-point indicator. The method was modified from previous research (Gumel et al., 2011). The volume of titrant required to neutralize the sample indicated the amount of residual lauric acid. The KOH volume

titrated was converted to lauric acid concentration using a linear calibration line that fitted the following equation (3):

$$C_{\text{lauric acid}} = 0.0089 \times V_{KOH} \times \text{molar mass lauric acid} \qquad \text{(eq. 3)}$$

where $C_{\text{lauric acid}}$: concentration of lauric acid (g L^{-1}); V_{KOH}: volume (mL) of the 0.1 M standardized KOH solution; molar mass of lauric acid: 200.32 g mol^{-1}. Equation (3) had a regression coefficient of 0.9914 and applied over a concentration range of 0.005 to 0.01 mol L^{-1} lauric acid. The standard calibration of lauric acid is shown in Figure 3.3.

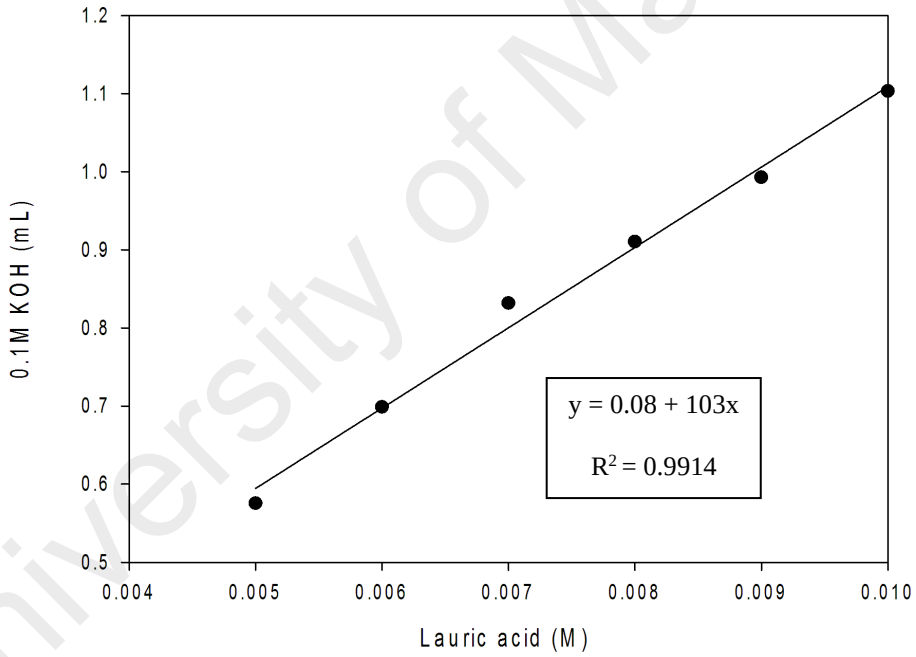


Figure 3.3: Standard calibration of lauric acid concentration.

3.2.5 *In vivo* depolymerization of intracellular mcl-PHA in production medium

Bacterial cells harvested from PHA production medium were recovered by centrifugation, washed and re-suspended into PHA depolymerization media. The cells containing intracellular mcl-PHA were incubated in carbon-free PHA production medium, MSM with NH_4^+ (1 g L^{-1}) or deprived of NH_4^+ for the control experiment (Yoon

& Choi, 1999). The cells were incubated under aerobic shaking condition at 25 ˚C, 200 rpm for 24 hours. Selected fermentation profiles were monitored for 120 hours. Approximately 10 mL of the culture was harvested every 24 hours. The bacterial mass following *in vivo* mcl-PHA depolymerization treatment was centrifuged and oven-dried to analyze the total biomass amount, mcl-PHA content and monomer composition. The supernatant was collected to profile exogenous depolymerization products of interest and residual ammonium in the medium.

3.2.5.1 Determination of residual NH_4^+ in the culture broth

The residual ammonium was determined from the cell-free culture liquid using thermometric titrator. The method is based on the measurement of heat released from exothermic reaction between analyte and titrant species. For sample preparation, the liquid medium containing residual ammonium was added with bromide/bicarbonate reagent. Samples were titrated with sodium hypochlorite (NaOCl) in the presence of *para*-formaldehyde as titration end-point indicator. The method was carried out as per recommended by the instrument manufacturer (http://www.metrohm.com/). The volume of titrant required to neutralize the sample indicated the amount of residual ammonium. The NaOCl volume was converted to ammonium concentration using a linear calibration line that fitted the following equation (4):

$$C_{\text{ammonium}} = 0.0027 \times V_{\text{NaOCl}} \times \text{molar mass ammonium} \quad \text{(eq. 4)}$$

where C_{ammonium}: concentration of ammonium (g L^{-1}); V_{NaOCl}: volume (mL) of the 0.1 mol L^{-1} standardized NaOCl solution; molar mass ammonium = 18.04 g mol^{-1}. Equation (4) had a regression coefficient of 1.000 and applied over a concentration range of 0.003 to 0.012 mol L^{-1} ammonium. The standard calibration of ammonium is shown in Figure 3.4.

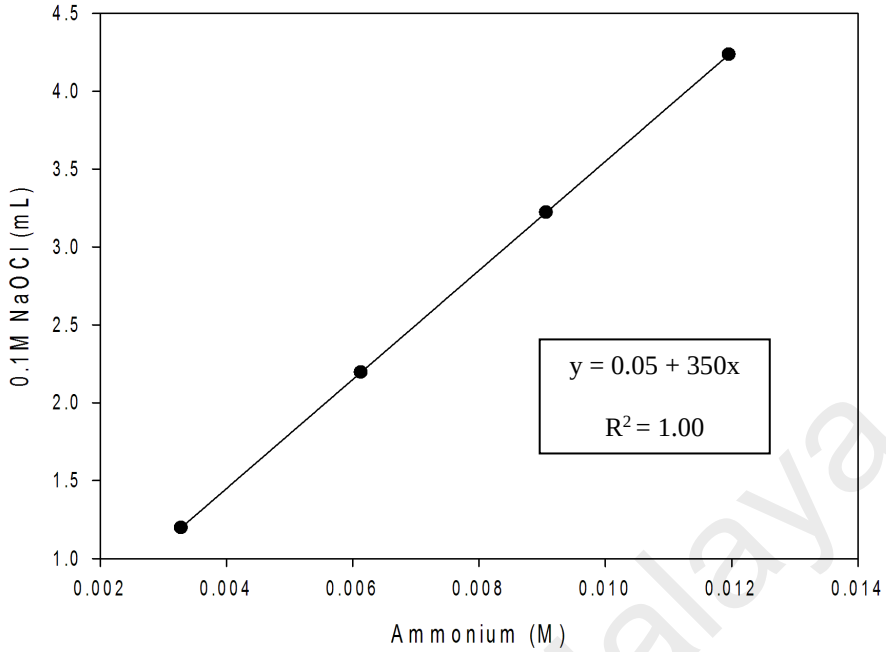


Figure 3.4: Standard calibration of ammonium concentration.

3.2.6 *In vivo* depolymerization of intracellular mcl-PHA in buffer media

Bacterial cells harvested from PHA production medium were recovered by centrifugation, washed and re-suspended into PHA depolymerization media. The cells containing intracellular mcl-PHA were incubated in different buffer solutions with different pH and ionic strength (*I*). The appropriate type of buffer, pH and *I* was selected to further determine the effects of temperature and buffer concentration. The cells were statically incubated for 48 hours and time profiling was carried out under optimal conditions. Approximately 10 mL of the culture was harvested every six hours or every twelve hours (for comparison study with *in vitro* depolymerization). The bacterial cells after *in vivo* depolymerization treatment was centrifuged and oven-dried to analyze the total biomass amount, mcl-PHA content and monomer composition. The supernatant was collected to profile exogenous depolymerization products of interest.

3.2.6.1 Effects of different types of buffers on *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001

Different types of buffers as listed in subsection 3.1.4.1 were used to study their effects on *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 for 24 hours incubation. The ensuing procedures were carried out as stated in section 3.2.6. The most suitable buffer was used for further experiments.

3.2.6.2 Effects of different temperatures on *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001

Different temperatures at 25, 30, 35 and 40 ºC were used to study their effects on *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 for 24 hours incubation. The ensuing procedures were carried out as stated in section 3.2.6. The most favorable temperature was used for further experiments.

3.2.6.3 Effects of different concentrations of buffer *on in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001

Different concentrations of buffer ranging from 0.025 until 0.2 M were used to study their effects on *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 for 24 hours incubation. The ensuing procedures were carried out as stated in section 3.2.6. The most suitable concentration of buffer was used for further experiments.

3.2.7 *In vitro* depolymerization of intracellular mcl-PHA in buffer media

The crude reaction sample (lysed cells) suspension was re-suspended into PHA depolymerization medium i.e. 0.2 M Tris-HCl buffer, pH 9, I = 0.2 M at 30 ºC was adopted from the previous *in vivo* depolymerization of intracellular mcl-PHA studies. The effects of different sonication time during the preparation of raw reaction sample on *in*

vitro depolymerization of intracellular mcl-PHA were studied to investigate the efficiency of depolymerization activity. The lysed cells were statically incubated for 48 hours and time profiling was carried out under optimal conditions. Approximately 10 mL of the culture was harvested every twelve-hour intervals. The lysed cells after *in vitro* depolymerization treatment was centrifuged and oven-dried (70 °C) to analyze the total lysed cells amount, mcl-PHA content and monomer composition. The supernatant was collected to profile exogenous depolymerization products of interest.

3.2.7.1 Sample preparation

Samples for *in vitro* depolymerization of intracellular mcl-PHA were prepared by lysing the bacterial cells containing intracellular mcl-PHA in ultrasonic bath (Elmasonic P 30H, Germany) sonicator. The cells suspension was sonicated at frequency 37 kHz, 30 % of power output (Ishak et al., 2016), with temperature controlled < 25 ºC for 120 minutes (except stated otherwise). Effect of sonication time was investigated to determine the minimum time for complete lysis of bacterial cells. The release of soluble protein was determined according to Bradford method (Bradford, 1976) for a range of sonication time.

3.2.7.2 Determination of protein concentration

The release of soluble protein was determined according to Bradford method with bovine serum albumin (BSA) as standard within of 0.25 to 2.0 mg mL^{-1} concentration (Bradford, 1976). 0.1 mL of sample was mixed with 3 mL Bradford Reagent. The samples were incubated at room temperature (30 ± 2 °C) for 5 - 45 minutes. The OD was determined at a 595 nm of wavelength. The protein dye complex is stable up to 60 minutes. The absorbance of the samples must be recorded before the 60 minutes time limit and within 10 minutes of each other. The protein concentration was determined by

comparison of the samples to the standard calibration prepared using the protein standards as shown in Figure 3.5. The protein concentration was calculated based on the relationship:

$$y = 0.5367x + 0.0269 \qquad \text{(eq. 5)}$$

where y is the OD reading at 595 nm and x is the calculated protein concentration (mg mL^{-1}). 1 unit of OD_{595} is equivalent to 1.81 mg mL^{-1} of protein concentration.

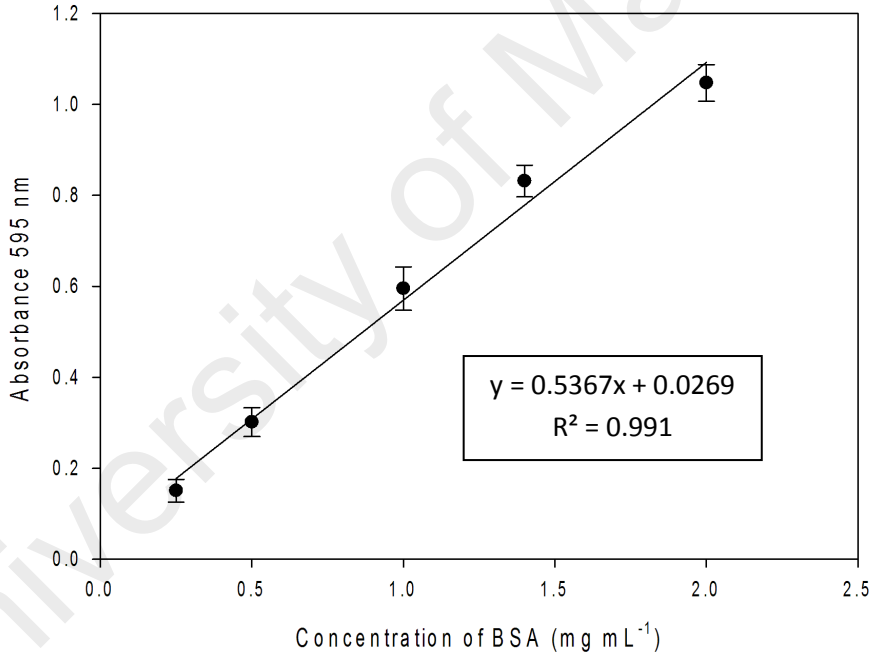


Figure 3.5: Standard calibration of protein concentration.

3.2.7.3 Effects of different sonication times on *in vitro* depolymerization of intracellular mcl-PHA

Different sonication times *viz.* 90, 120 and 150 minutes were used to study the effects of different sonication times on *in vitro* depolymerization of intracellular mcl-PHA. The raw reaction sample with different sonication times were collected and re-

suspended in buffer solution (0.2 M Tris-HCl buffer, pH 9, I = 0.2 M). The ensuing procedures were carried out as stated in section 3.2.7. The most suitable sonication time was used for further experiments.

3.2.8 Enzyme activity

One unit (1 U) of intracellular enzymatic depolymerization activity is defined as the total enzyme action that liberates 1 μmol per minute the monomers available within the mcl-PHA. Bacterial cells containing mcl-PHA exposed to thermal treatment at 70 ˚C for 10 minutes were used as control for depolymerization experiments.

3.2.9 PHA extraction

Mcl-PHA extraction was carried out by re-suspending the dried cells in dichloromethane (DCM) (Merck, USA) and then refluxed for 4 hours at 60 ± 5 ˚C. Buchner filter funnel with sintered glass was used to filter the extracted mcl-PHA. Filtrate was then concentrated under vacuum in a rotary evaporator at 60 ˚C and 70 rpm until about 1/6 of total volume. A beaker containing excess cold methanol (Merck, USA) was used to precipitate mcl-PHA, in the volume ratio of 1:4. Purification steps were repeated three times by re-dissolving the product in DCM followed by re-precipitation in cold methanol. Mcl-PHA content (% of total biomass) was determined based on its gravimetric analysis against dried total biomass as reported previously (Annuar et al., 2007; Gumel et al., 2012).

3.2.10 Quantitative analysis

3.2.10.1 Mcl-PHA content and monomer composition

The mcl-PHA content and monomer composition of bacterial cells before and after depolymerization were determined using gas chromatography (GC) (Trace GC

Ultra, Thermo Scientific, Italy) equipped with a flame ionization detector (FID) and a fused silica capillary column (30 m length × 0.32 mm internal diameter, 0.25 µm film) (Supelco, SPB-TM1, USA). Methanolysis was used to prepare sample for GC analysis in order to produce methyl ester derivatives of the PHA monomer (Randall, 2008). During methanolysis, the backbone of the polymer will be broken at the ester bond with the presence of sulfuric acid as the catalyst. Methyl groups will be added to the exposed carboxyl group ends (Ishak, 2014). The methanolysis was performed using a modified method according to Braunegg et al. (1978). Approximately 10 to 20 mg of dry cells were subjected to methanolysis in the presence of 2 mL DCM with 2 mL methanol: sulfuric acid [85 %:15 % *v/v*]. The reaction mixture was incubated at 100 ˚C for 140 minutes. The organic layer containing the reaction products was separated, dried over Na_2SO_4, and analyzed by GC. Benzoic acid methyl ester (Fluka, Germany) was used as the internal standard. 1.0 µL of methanolyzed PHA sample was injected into the GC machine using splitless injection mode. Helium was used as the carrier gas at a flow rate of 2 mL min^{-1}. The column oven temperature was programmed at 50 ˚C ramped up at a rate of 10 ˚C min^{-1} to 280 ˚C, and held at this temperature for 2 min. The temperatures of injector and detector were set at 200 ˚C and 280 ˚C, respectively. The PHA standard monomers of methyl hydroxyalkanoates (Larodan, Sweden & Sigma Aldrich, Germany) were used as representative references for peak retention time and standard calibration for each monomer concentration. Mcl-PHA content (%) was obtained by summing up all individual monomers present and given as mcl-PHA weight (mg) per dried total biomass (mg) (Gumel et al., 2012; Razaif - Mazinah et al., 2016).

3.2.10.2 Identification of mcl-PHA depolymerization products

The supernatant from *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA was collected, acidified using concentrated HCl to pH 1.0 and extracted using

dichloromethane (DCM) (Merck, USA) for detecting the presence of exogenous depolymerization products of interest using gas chromatography-mass spectrometry (GCMS). GCMS analysis was recorded on Shimadzu GC coupled to Shimadzu QP 2010 Ultra MS (Shimadzu, Model 2010, Japan) operated in electron impact ionization mode (70 eV) with RTX-5 column (Restek Corporation, USA) (30 m long × 0.25 mm internal diameter, 0.25 µm film thickness). The water-soluble compounds of exogenous direct products from both *in vivo* and *in vitro* depolymerizations were subjected to silylation for GCMS analysis. Silylation is the most established derivatization method as it is readily volatizes the non-volatile sample by the addition of silyl groups to the free –OH groups. The silylated derivatives are more volatile and more stable for GC analysis (Orata, 2012). Approximately 8.0 mg of dried supernatant sample were filled with 100 µL N, N–bis(trimethyl-silyl)trifluoro-acetamide (BSTFA) (Merck, USA) in a clean and dry reaction vial. The mixture was vortexed for 5 minutes, incubated for 15 minutes at 70 °C (Allen et al., 2012) and filtered (0.22 µm PTFE membrane filter) before automatic injection into the GCMS machine. 1.0 µL of silylated samples was injected into the GCMS machine using splitless injection mode. The inlet temperature was 200 °C. The column oven temperature profile was pre-set as follows: 90 °C for 0 min then ramped up to 280 °C at a rate of 5 °C min^{-1} and held for 10 min. Helium was used as carrier gas at a flow rate of 1.5 mL min^{-1} (Aris et al., 2016).

3.2.10.3 Molecular weight determination

Determination of molecular weights of mcl-PHA before and after depolymerizations was carried out using GPC (Agilent, LC 1220, USA) instrument equipped with a refractive index detector (Model 1260). The machine was equipped with Mixed-D gel column (7.8 mm internal diameter × 300 mm) connected to MiniMix-D gel column (5 mm internal diameter × 25 mm). PHA sample was dissolved in tetrahydrofuran

(THF) (Merck, USA) at a concentration of 2.0 mg mL^{-1} and was filtered through 0.22 µm PTFE filter. Then, 100 µL of the sample was injected at 40 ˚C with THF as mobile phase at a flow rate of 1.0 mL min^{-1}. The chromatogram was recorded relative to the calibration curve of standard mono-disperse polystyrenes (162, 380, 1020, 1320, 2930, 6770, 13030, 29150, 51150, 113300, 215000, and 483400 g mol^{-1}) (Agilent, EasiVials, USA). The GPC measurements were used to characterize the average weight molecular weight (M_w), number average molecular weight (M_n), and the polydispersity index (PDI) of polymer (Aris et al., 2016).

3.2.10.4 Thermal properties

Thermal properties of mcl-PHA before and after depolymerizations were determined using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC):

i) TGA analysis was performed using Perkin Elmer TGA 4000 instrument (Perkin Elmer, USA). Approximately, 8 to 10 mg of the PHA sample was loaded onto the ceramic crucible pan. The sample was heated from 30 ˚C to 800 ˚C at a rate of 10 ˚C min^{-1} under a nitrogen flow rate of 20 mL min^{-1}.

ii) DSC analysis was performed using DSC Q20 machine (TA Instrument, UK) analysis. Approximately 5 mg of sample was prepared in an aluminum pan. Analysis was performed at a programmed temperature range of 5 ˚C to 300 ˚C with a heating rate of 10 ˚C min^{-1} under nitrogen gas flow rate 50 mL min^{-1} at a head pressure 1.5 bars. The endothermic peaks of DSC thermogram were taken as the melting temperature (T_m).

3.2.10.5 ^{1}H NMR analysis

^{1}H-NMR analysis was performed using FT-NMR AVANCE III spectrometer (Bruker, New Zealand) at 400 MHz. Approximately 5 mg PHA sample was dissolved in 2 mL deuterated chloroform ($CDCl_3$) (Merck, USA) and filtered into NMR tube using borosilicate glass syringe equipped with 0.22 µm PTFE filter. Tetramethylsilane (TMS) was used as internal reference.

3.2.10.6 Statistical analysis

All measurements were conducted in triplicate. Variance analysis was used to evaluate the significance of results, and $p < 0.05$ was considered to be statistically significant.

3.2.11 Qualitative analysis

3.2.11.1 Observation of bacterial cell morphology using light microscopy

The morphology of bacterial cell was observed under light microscope (Motic, BA200, USA) with oil immersion lens equipped with Megapixel Moticam 2300 digital camera for visualization on a computer and to capture the magnified image. A loopful of the liquid culture was transferred onto the surface of a clean glass slide, and spread over a small area as thin layer. The cell layer was fixed on the glass slide by passing it briefly through the Bunsen burner flame for two or three times without exposing it directly to the flame. Then, the slide was observed under the microscope.

3.2.11.2 Observation of bacterial cell surface morphology using field emission scanning electron microscopy (FESEM)

The surface morphology of bacterial cell before and after *in vivo* depolymerization of intracellular mcl-PHA was observed under three-dimensional view using field

emission scanning electron microscopy (FESEM) (Quanta, FEG 450, USA). Hexamethyldisilazane (HMDS) method was used for the samples preparation. First, the samples were fixed using McDowel-Trump solution (consisting of 4 % formalin and 1 % glutaraldehyde) prepared in 0.1 M phosphate buffer (pH 7.2) at least for 2 hours. The re-suspended samples were then washed twice using 0.1 M phosphate buffer before the post-fixation using 1 % *v/v* osmium tetroxide prepared in the phosphate buffer for 1 hour. After that, the samples were washed twice with distilled water followed by a dehydration process (50 % *v/v* ethanol 10 minutes, 75 % *v/v* ethanol 10 minutes, 95 % *v/v* 10 minutes, 100 % *v/v* ethanol 10 minutes (repeat once), HMDS 10 minutes). After the dehydration process, the samples were placed in dessicator to dry at room temperature. The dried samples were mounted onto a FESEM specimen stub with a double-sided sticky tape to be coated with gold particles before viewing under the FESEM.

3.2.11.3 Observation of bacterial cell morphology using confocal laser microscopy

The cell morphology before and after being lysed using bath sonicator was observed under bright field and fluorescent imaging using confocal laser microscopy (Leica Tcs Sp5 Ii, Germany). Nile red (Sigma Aldrich, Germany) was used to stain intracellular mcl-PHA granules. It is the finest fluorescent dye used for intracellular lipid-like PHA allowing for observation under fluorescence microscopy (Rumin et al., 2015). The Nile red stock solution (5 µg mL^{-1}) was prepared by dissolving the dye in DMSO (Fluka, Germany). 20 µL of Nile red from stock was mixed with 1.0 mL bacterial suspension before and after depolymerization and incubated for 15 minutes. Then the bacterial suspension was centrifuged (2800 × *g*, 30 °C) for 3 minutes and re-suspended in 1.0 mL distilled water (Amirul, 2007). 20 µL of bacterial suspension dyed with Nile red was pipetted onto a glass slide, spread over a small area and covered with a glass slip cover for microscopic observation.

3.2.12 Calculations

i. $\%\ PHA\ content = \frac{PHA\ mass}{Total\ biomass\ (PHA\ mass + PHA{-}free\ cell\ mass)} \times 100$ (eq. 6)

ii. $\%\ PHA\ depolymerization = \frac{(PHA\ mass\ before - PHA\ mass\ after\ depolymerization)}{PHA\ mass\ in\ biomass\ before\ depolymerization} \times 100$ (eq. 7)

iii. Volumetric rate of depolymerization (g L^{-1} h^{-1}) = $\frac{\Delta\ PHA\ mass}{\Delta\ time}$ (eq. 8)

iv. Ionic strength (I) = $\frac{1}{2}\sum_{i=1}^{n} C_i Z_i^2$ (eq. 9)

where C = molar concentration ion i; Z = charge no. of ion i

v. Percentage of liberated monomer (µmol L^{-1}) = $\frac{M_0 - M_t}{M_0} \times 100$ (eq. 10)

where M_0 = total mass of monomer; M_t = monomer mass at time t.

vi. Depolymerization activity (U L^{-1}) = $\frac{mol\ of\ liberated\ monomer}{time \cdot volume}$ (eq. 11)

where unit activity (U) is expressed in µmol min^{-1} per liter of reaction mixture.

vii. $\%\ Yield = \frac{Actual\ Yield}{Expected\ Yield} \times 100\%$ (eq. 12)

viii. Dilution factor (DF) = $\frac{vol.\ of\ sample + vol.\ of\ water}{vol.\ sample} \times DF\ before$ (eq.13)

ix. C/N calculation example for lauric acid with $NaNH_4HPO_4$ $4H_2O$ (C/N 20):

Calculation for mol of N in 3.5 g of $NaNH_4HPO_4$ $4H_2O$ in 1 L MSM,

Molar mass $NaNH_4HPO_4$ $4H_2O$ = 209.07 g mol^{-1}

Molar mass no. of N = 14.01 × 1 = 14.01 g mol^{-1}

Mass of N = $\frac{molar\ mass\ no.of\ N}{molar\ mass\ NaNH4HPO4.4H2O} \times total\ mass\ NaNH_4HPO_4\ 4H_2O$ (eq. 14)

= 0.234 g

Mol of N = $\frac{mass\ of\ N}{molar\ mass\ of\ N}$ (eq. 15)

= 0.017 mol

Thus, mol of C (C/N 20) in 1 L MSM can be calculated by:

0.017 mol N × 20 = 0.335 mol C

C/N 20 is equivalent to 0.335 mol C: 0.017 mol N

From this, lauric acid mass in gram needed in 1 L MSM was calculated,

Molar mass lauric acid ($C_{12}H_{24}0_2$) = 200.32 g mol^{-1}

Molar mass no. of C = 12.01 × 12 = 144.12 g mol^{-1}

Mass of C = $mol\ of\ C \times atomic\ mass\ of\ C$ (eq. 16)

= 0.335 mol × 12.01 = 4.02 g

Total mass lauric acid was calculated using the following equation:

$\frac{molar\ mass\ no.\ of\ C}{molar\ mass\ lauric\ acid} \times total\ mass\ lauric\ acid\ (X\ gram) = mass\ of\ C$ (eq. 17)

X gram is equivalent to 5.58 g in 1 L @ 0.56 g in 100 mL MSM

All carbon sources were similarly calculated. For SPKO (*MW* 217, Cocks & van Rede, 1996), similar calculation was made that taking into account that it contained approximately 48 % (*w/w*) of lauric acid.

x. Monomer amount and composition by GC analysis.

The relative response factor of a particular 3-hydroxyacid methyl ester (R_{fx}) to

the internal standard was calculated according to Annuar (2004):

$$R_{fx} = [A_x]/[A_{IS}] \times [C_{IS}]/[C_x] \quad \text{(eq. 18)}$$

where A_x is the 3-hydroxyacid methyl ester area;

A_{IS} is the methyl benzoate area;

C_{IS} is the methyl benzoate concentration; and

C_x is the 3-hydroxyacid methyl ester concentration.

To calculate the concentration of 3-hydroxyacid methyl ester in a test sample (for example C_8), the relationship given below was used (Annuar, 2004):

$$\text{Concentration } C_8\ (\mu g\ mL^{-1}) = (\text{Area } C_8 \times \text{Concentration}_{IS}) / (\text{Area}_{IS} \times R_{fC8}) \quad \text{(eq. 19)}$$

where Area C_8 is peak area for the C_8 monomer;

Area_{IS} is peak area for the methyl benzoate;

$\text{Concentration}_{IS}$ is the amount of methyl benzoate per mL DCM; and

R_{fC8} is the relative response factor for the C_8-methyl ester.

Total polymer amount was obtained by summing up all the individual monomers present, and the mcl-PHA fraction was calculated from the total biomass dry weight used for methanolysis.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Microbial Biosynthesis of Intracellular Medium-Chain-Length Poly-3-hydroxyalkanoates

Biosynthesis of mcl-PHA by *P. putida* Bet001 was carried out to obtain relatively high intracellular amount up to 50 wt% of DCW and above. Sufficient amount is essential for reliable monitoring of *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA. Different parameters such as the effects of different carbon sources, C/N ratio, cell concentration and incubation time were studied to enhance biomass growth and mcl-PHA biosynthesis. The highest biomass (8 g L^{-1}) and PHA yield (> 50 wt%) from total biomass were achieved when the microorganism was cultured into PHA production medium using C/N 20 of lauric acid (C_{12}) as a sole carbon and energy source at 1 g L^{-1} of initial cell concentration for 48 hours cultivation (Figure 4.1a-d).

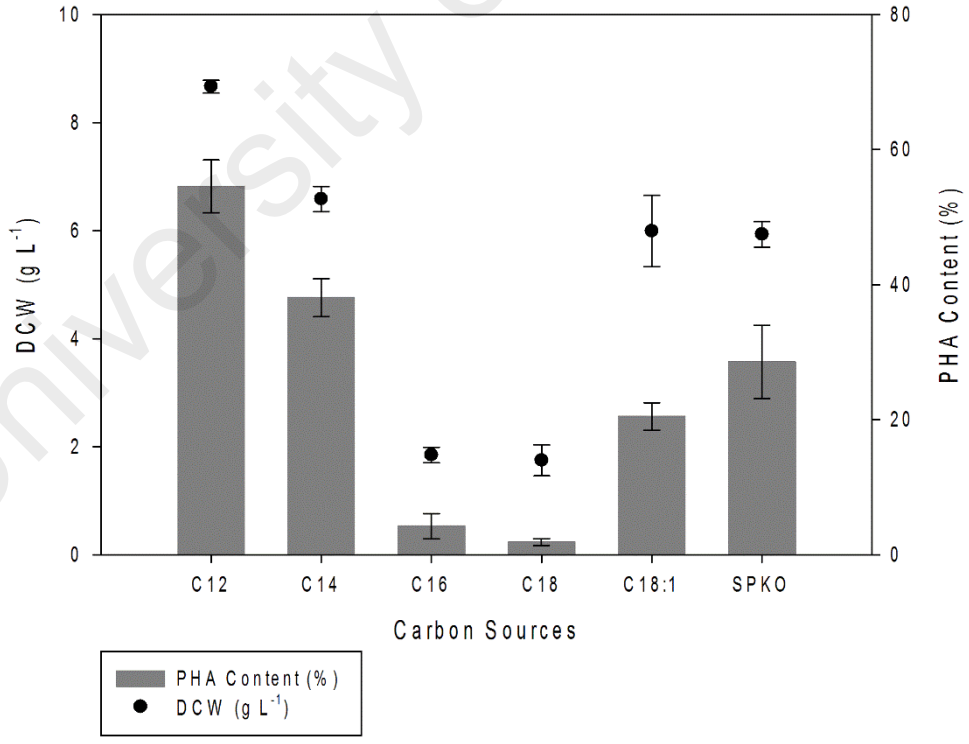


Figure 4.1a: Effects of various carbon substrates on DCW and PHA content produced by *P. putida* Bet001. Values are means of three replications ± standard deviation.

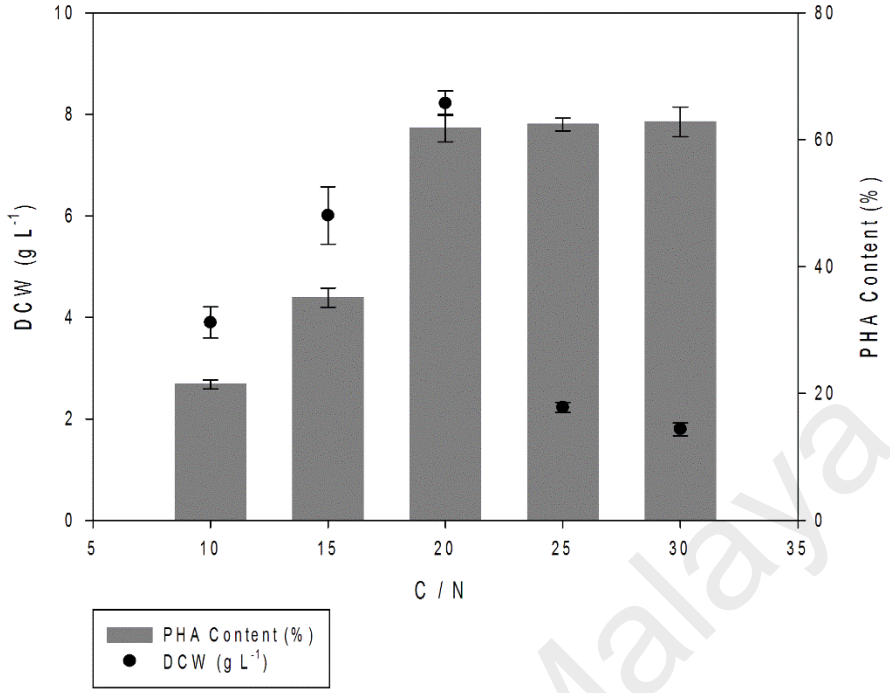


Figure 4.1b: Effects of different C/N ratios on DCW and PHA content produced by *P. putida* BET001. Values are means of three replications ± standard deviation.

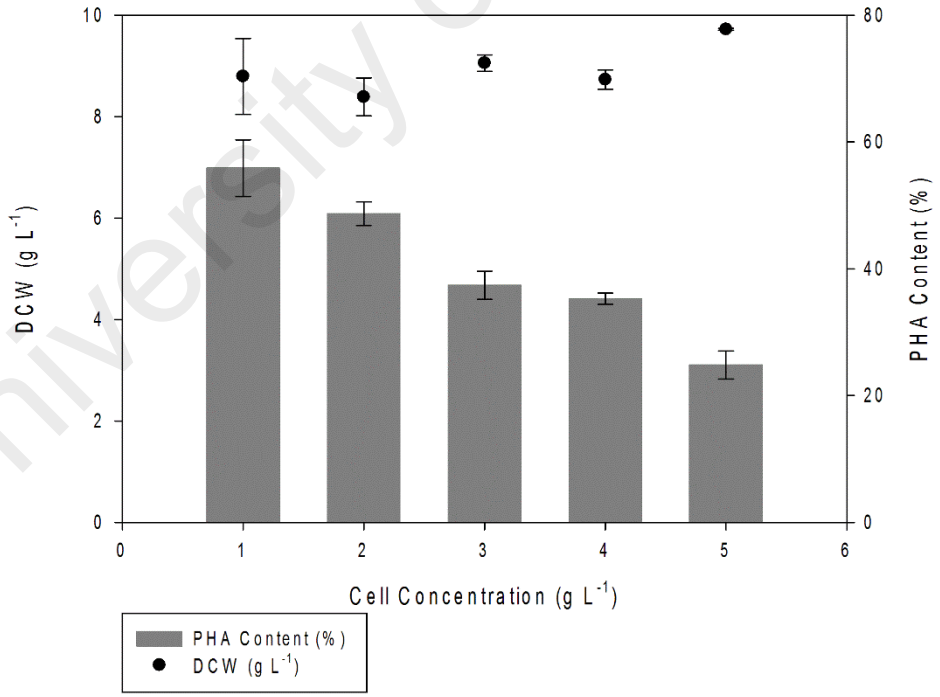


Figure 4.1c: Effects of different initial cell concentrations on DCW and PHA content produced by *P. putida* BET001. Values are means of three replications ± standard deviation.

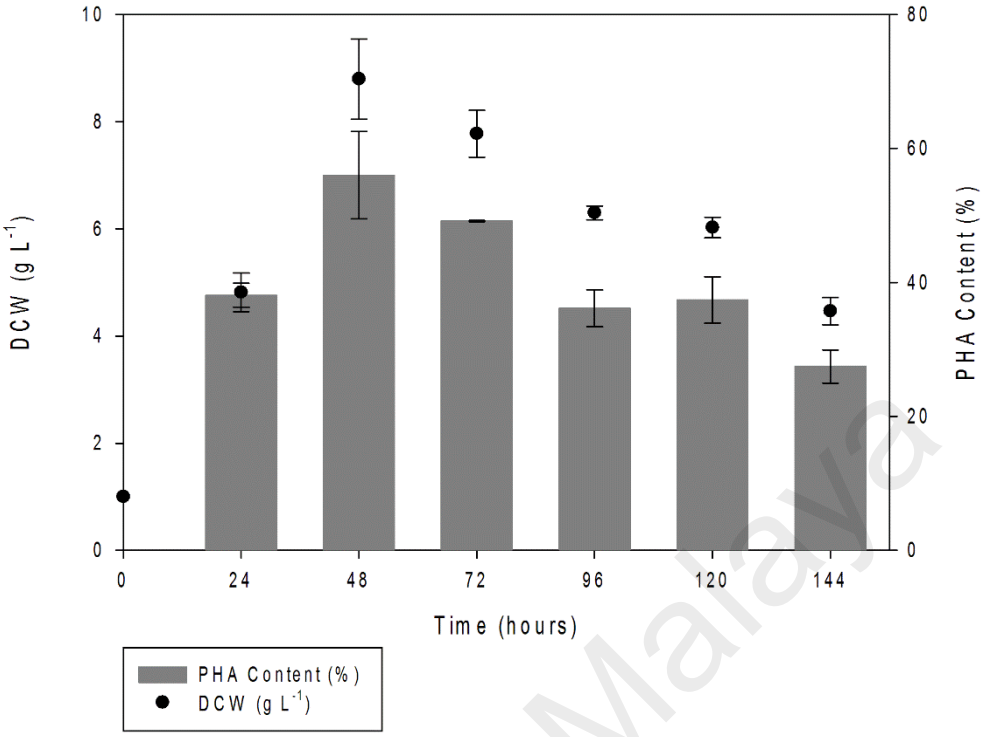


Figure 4.1d: Effects of different incubation times on DCW and PHA content produced by *P. putida* BET001. Values are means of three replications ± standard deviation.

Lauric acid (C_{12}) is a relatively cheap fatty acid that can be found as waste product in vegetable oil production and suitable for PHA fermentation process (Ballistreri et al., 2001). Under the favorable fermentation conditions stated, six independent experiments (n = 6) were conducted to determine the consistency of monomer composition identities and mol fractions in intracellular mcl-PHA prior to depolymerization studies. The mcl-PHA produced comprised of even number carbon atom chain length monomers: 3-hydroxyhexanoate (3HHx) (C_6) (6.1 ± 2.0 mol %), 3-hydroxyoctanoate (3HO) (C_8) (40.1 ± 2.7 mol %), 3-hydroxydecanoate (3HD) (C_{10}) (32.8 ± 2.8 mol %) and 3-hydroxydodecanoate (3HDD) (C_{12}) (21.0 ± 3.2 mol %) (Figure 4.1e). The determined monomer composition agreed with previous research when the supplied carbon source has linear 6- to 12-carbon atoms whereby the monomers produced were of the same length with the carbon source or has been shortened by two, four or six carbon atoms *via*

β-oxidation pathway for PHA biosynthesis (Ballistreri et al., 2001). The cultivation conditions found favorable for producing high PHA content in *P. putida* Bet001 were reproduced as closely as possible to obtain culture preparation intended for *in vivo* depolymerization of intracellular mcl-PHA studies. The residual lauric acid was also estimated. It can be seen that within 24 hours of cultivation, the bacterial culture almost completely utilized the carbon source supplied while the mcl-PHA content remained at approximately 50 wt% for the next 48 hours (Figure 4.1f). Thus, the most appropriate time range to harvest the culture for subsequent *in vivo* depolymerization studies would be within 48 to 72 hours of cultivation. After 72 hours, the polymer content started to decrease due to the intracellular depolymerization of mcl-PHA following external carbon source depletion (Handrick et al., 2001; Tokiwa & Ugwu, 2007).

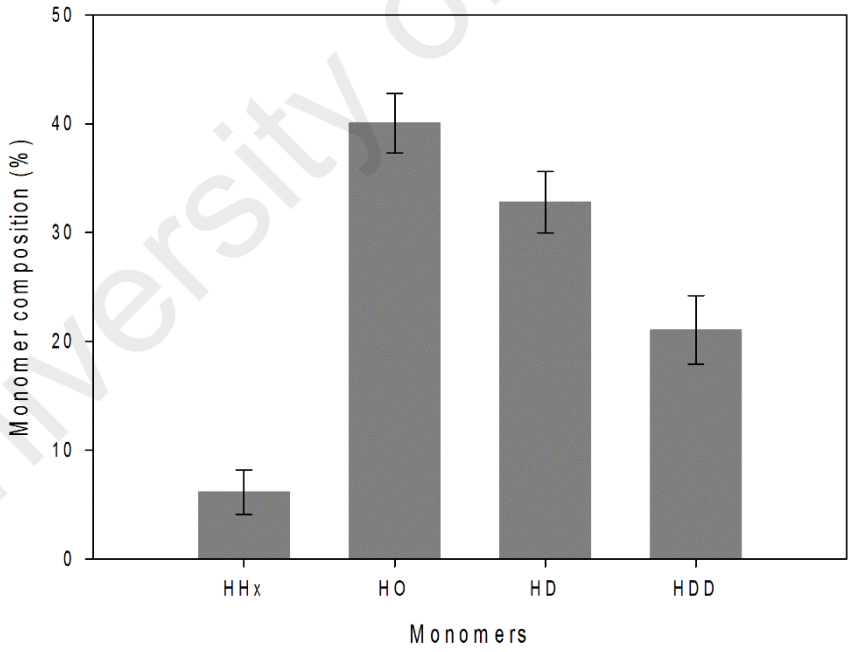


Figure 4.1e: Monomer composition of mcl-PHA synthesized in lauric acid as a carbon source. Values are means of three replications ± standard deviation. (*HHx: C_6; HO: C_8; HD: C_{10}; HDD: C_{12}*).

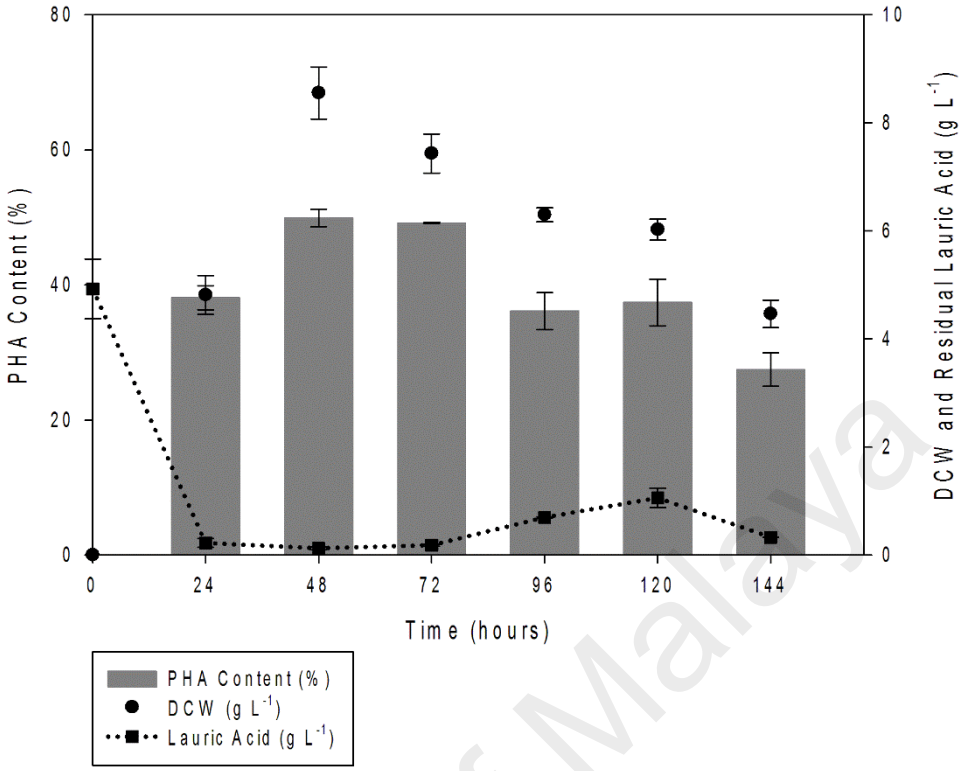


Figure 4.1f: Determination of residual fatty acid during mcl-PHA biosynthesis. Values are means of three replications ± standard deviation.

4.2 *In vivo* Depolymerization of Intracellular Medium-Chain-Length Poly-3-hydroxyalkanoates

4.2.1 *In vivo* depolymerization of intracellular mcl-PHA in production medium

Three different types of production media were used to compare the extent of PHA depolymerization after 24 hours incubation as shown in Table 4.1a. The percentage of intracellular PHA depolymerization by the cell culture in the original PHA production medium (MSM 1) without being transferred to other medium showed no significant difference with the culture that was transferred to fresh mineral medium without carbon and ammonium sources (MSM 2) (depolymerization percentage 23.6 to 24.4 wt%). This was in contrast to the carbon source-omitted medium supplied with high ammonium concentration (MSM 3) where higher percentage of PHA depolymerization was observed at 44.3 ± 2.0 wt% after 24 hours incubation.

The presence of ammonium was hypothesized to play a role in the more active intracellular depolymerization of mcl-PHA by the culture possibly through higher turnover of depolymerase protein. As a result, the depolymerization percentage of mcl-PHA was higher compared to the medium devoid of ammonium (Yoon & Choi, 1999). For MSM 3, extended time profiles for *in vivo* depolymerization of intracellular mcl-PHA are shown in Figure 4.2a and Table 4.1b. Almost 90 wt% of mcl-PHA in bacterial cell culture was degraded after 120 hours incubation. The average initial content of mcl-PHA in the cell population was 55.1 ± 1.9 wt%. DCW decreased from 5.4 to 2.2 g L^{-1} after 120 hours incubation. However, the residual ammonium concentration only decreased from 1 to 0.87 g L^{-1} after 120 hours (Figure 4.2a).

Table 4.1a: Comparison of *in vivo* depolymerization of intracellular mcl-PHA in different mineral media.

Medium	**Initial PHA (wt%)**	**Final PHA (wt%)**	**Initial DCW (g L^{-1})**	**Final DCW (g L^{-1})**	**PHA de-polymerized (%)**	**Average volumetric rate of depolymerization (g L^{-1} h^{-1})**
Mineral medium after 72 hours biosynthesis in the original medium (MSM 1)	55.9 ± 1.30	49.1 ± 0.10	8.55 ± 0.48	7.43 ± 0.36	23.6 ± 0.90	0.05 ± 0.00
Fresh mineral medium lacking carbon source and ammonium (MSM 2)	48.6 ± 0.50	41.9 ± 0.50	8.01 ± 0.11	7.02 ± 0.01	24.4 ± 1.10	0.04 ± 0.01
Fresh mineral medium lacking carbon source + 1 g L^{-1} ammonium (MSM3)	48.7 ± 1.90	34.3 ± 2.50	7.82 ± 0.38	6.18 ± 0.23	44.3 ± 2.00	0.07 ± 0.01

*Values are means of three replications ± standard deviation.

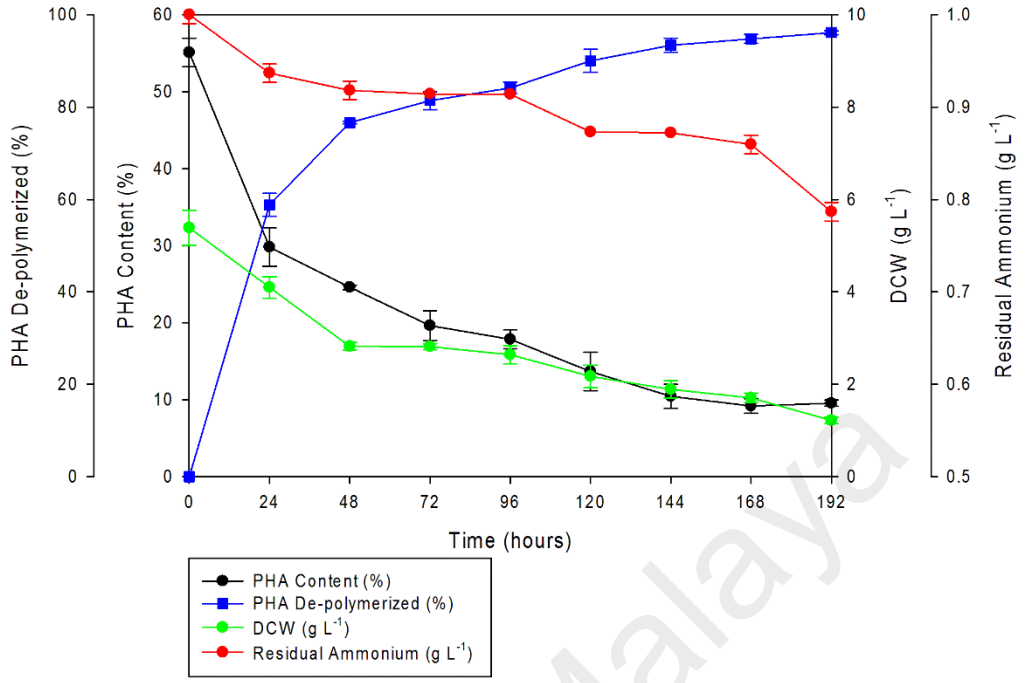


Figure 4.2a: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in mineral medium lacking carbon source supplied with 1 g L^{-1} ammonium (MSM 3). Values are means of three replications ± standard deviation.

Table 4.1b: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in mineral medium lacking carbon source supplied with 1 g L^{-1} ammonium (MSM 3).

Time (hour)	DCW (g L^{-1})	PHA content (wt%)	PHA content (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
0	5.38 ± 0.38	55.08 ± 1.87	2.97 ± 0.15	0	0
24	4.10 ± 0.23	29.82 ± 2.49	1.22 ± 0.12	58.84 ± 2.49	1.75 ± 0.01
48	2.82 ± 0.08	24.59 ±0.28	0.69 ± 0.05	76.63 ± 0.28	2.27 ± 0.01
72	2.82 ± 0.07	19.62 ± 1.93	0.55 ± 0.01	81.38 ± 1.93	2.42 ± 0.02
96	2.64 ± 0.20	17.84 ± 1.23	0.47 ± 0.02	84.13 ± 1.23	2.50 ± 0.02
120	2.17 ± 0.24	13.67 ± 2.51	0.30 ± 0.03	89.99 ± 2.51	2.67 ± 0.01
144	1.89 ± 0.19	10.44 ± 1.55	0.20 ± 0.02	93.35 ± 1.55	2.80 ± 0.04
168	1.71 ± 0.10	9.18 ± 0.97	0.16 ± 0.01	94.72 ± 0.97	2.81 ± 0.03
192	1.22 ± 0.07	9.55 ± 0.42	0.12 ± 0.01	96.08 ± 0.42	2.85 ± 0.10

*Values are means of three replications ± standard deviation.

The consumption of ammonium was not observed to be as dramatic as the previous research by Yoon and Choi (1999) on *in vivo* depolymerization of intracellular P(3HB) where it was depleted after certain time. The physiological function of accumulated PHA in bacteria is to provide for energy and carbon metabolism during starvation period as prolonged survival strategy (Jendrossek, 2009). Figure 4.2b showed the amount of different monomers liberated from depolymerization activities (µmol L^{-1}) during *in vivo* depolymerization of intracellular mcl-PHA in MSM 3. The cells started immediately in the depolymerization of mcl-PHA with simultaneous release of all monomers within the initial 24 hours incubation period, and gradually continued to do so until it reached a plateau at 72 hours for C_6 and C_{12}, and at 96 hours for C_8 and C_{10}. Monomer liberation had ceased afterwards.

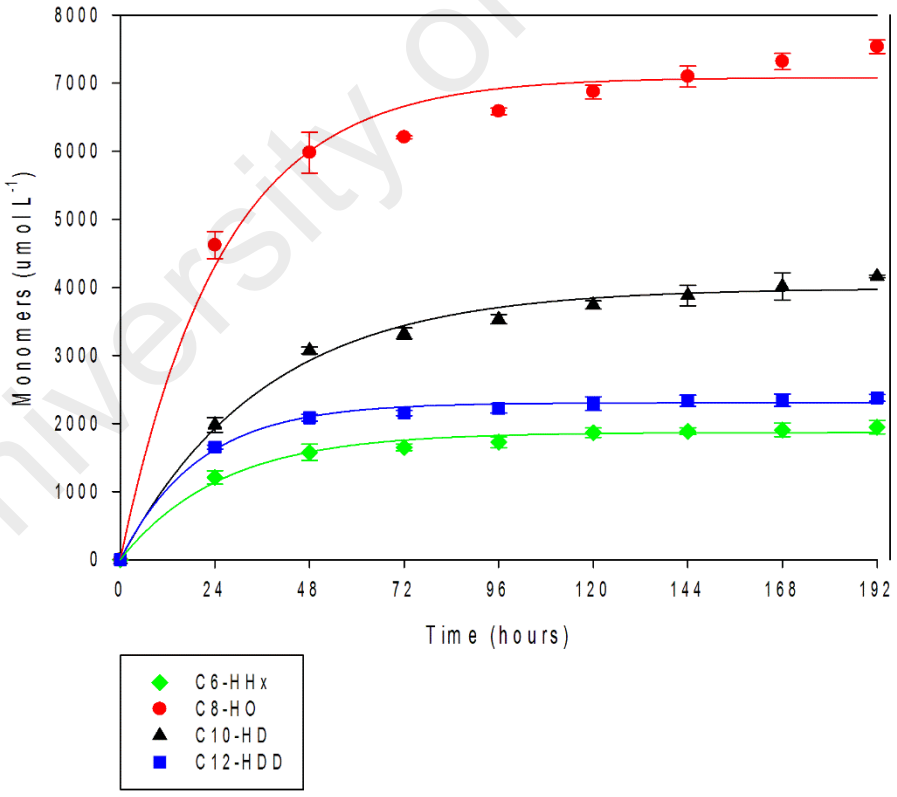


Figure 4.2b: Amount of different monomers liberated during *in vivo* depolymerization of intracellular mcl-PHA in mineral medium lacking carbon source supplied with 1 g L^{-1} ammonium (MSM 3). Values are means of three replications ± standard deviation.

The depolymerization activities were a direct reflection of the initial mol percentage of monomer distribution with the highest rate of depolymerization for a specific monomer was observed for C_8 followed by C_{10}, C_{12} and C_6 (Table 4.1c). The rate of mcl-PHA depolymerization was 0.07 g L^{-1} h^{-1} equivalent to 6.58 ± 0.09 U L^{-1} apparent depolymerization activity within 24 hours of incubation (Table 4.1c). The rate of depolymerization of different monomers ranged from 0.01 to 0.03 g L^{-1} h^{-1} corresponding to apparent depolymerization activities ranging from 0.84 to 3.21 U L^{-1}.

Table 4.1c: Average volumetric rate of *in vivo* depolymerization of intracellular mcl-PHA and apparent enzymatic depolymerization activities towards different monomers in mineral medium lacking carbon source supplied with 1 g L^{-1} ammonium (MSM 3).

	Mcl-PHA	**C_6-HHx**	**C_8-HO**	**C_{10}-HD**	**C_{12}-HDD**
Initial (g L^{-1})	2.97 ± 0.23	0.26 ± 0.02	1.27 ± 0.10	0.89 ± 0.08	0.55 ± 0.05
Final (g L^{-1})	1.22 ± 0.17	0.10 ± 0.00	0.53 ± 0.06	0.49 ± 0.04	0.17 ± 0.02
Average volumetric rate of depolymerization (g L^{-1} h^{-1})	0.07 ± 0.01	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Apparent depolymerization activity (U L^{-1})	6.58 ± 0.09	0.84 ± 0.01	3.21 ± 0.05	1.38 ± 0.02	1.15 ± 0.01

*Values are means of three replications ± standard deviation.

4.2.2 *In vivo* intracellular depolymerization of mcl-PHA in buffer medium

It has been reported that by incubating the culture of PHA-containing bacteria in a suitable buffer with appropriate environmental conditions is likely to promote high depolymerization activities (Lee et al., 1999; Ren et al., 2005). In this study, different buffer media were used to investigate *in vivo* depolymerization of intracellular mcl-PHA. Bacterial cell culture after 48 to 72 hours of cultivation in the production medium were collected by centrifugation and re-suspended in different buffer solutions. The percentage of intracellular mcl-PHA depolymerization was calculated from GC analysis before and

after 24 hours incubation. Figure 4.3a and Table 4.2a showed that buffer solutions with different pH and *I* had significant effects towards depolymerization activities. The highest amount of *in vivo* depolymerization of intracellular mcl-PHA was obtained with 0.2 M Tris-HCl buffer with initial pH 9, I = 0.2 M (77.9 ± 1.0 wt%) compared with 0.2 M glycine-NaOH buffer with the same initial pH 9, I = 0.4 M (70.2 ± 3.3 wt%). At initial pH 2 until 8, *in vivo* depolymerization of intracellular mcl-PHA was found to be lower i.e. within the range of 43.9 to 64.8 wt% in the acidic and neutral conditions compared to basic conditions. Furthermore, the depolymerization activities were observed to be decreased (68.9 - 66.0 wt% of PHA depolymerization) in the extreme basic condition at pH 10 and 11. The finding was in agreement with previous reports that the optimum pH for depolymerization activity was observed within moderate alkaline range (Ren et al., 2005; Ruth et al., 2007). Wang and co-workers reported that *Pseudomonas putida* GP01 had lost most of its accumulated mcl-PHA after alkaline incubation (Wang et al., 2007). It was clear from Figure 4.3a and Table 4.2a there is a congruity vis-à-vis to specific buffer type, molarity, pH and *I* values favorable as a medium for enhanced *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 whole cells. The reason behind the observation is yet to be fully elucidated. Other investigation has also shown that ionic strength of the buffer medium could significantly affect the intracellular depolymerization activities when studied as appropriate ratio of biopolymer inclusion bodies to buffer (Foster et al., 1999). In this study, 0.2 M Tris-HCl, pH 9 and I = 0.2 M exhibited highest intracellular depolymerization activities by *P. putida* Bet001 whole cells without the need to include any promoter e.g. magnesium chloride as previously reported by Foster et al. (1996). Hence, 0.2 M Tris-HCl was selected as a starting point for further research as it could provide the favorable conditions for *in vivo* intracellular mcl-PHA depolymerization activities.

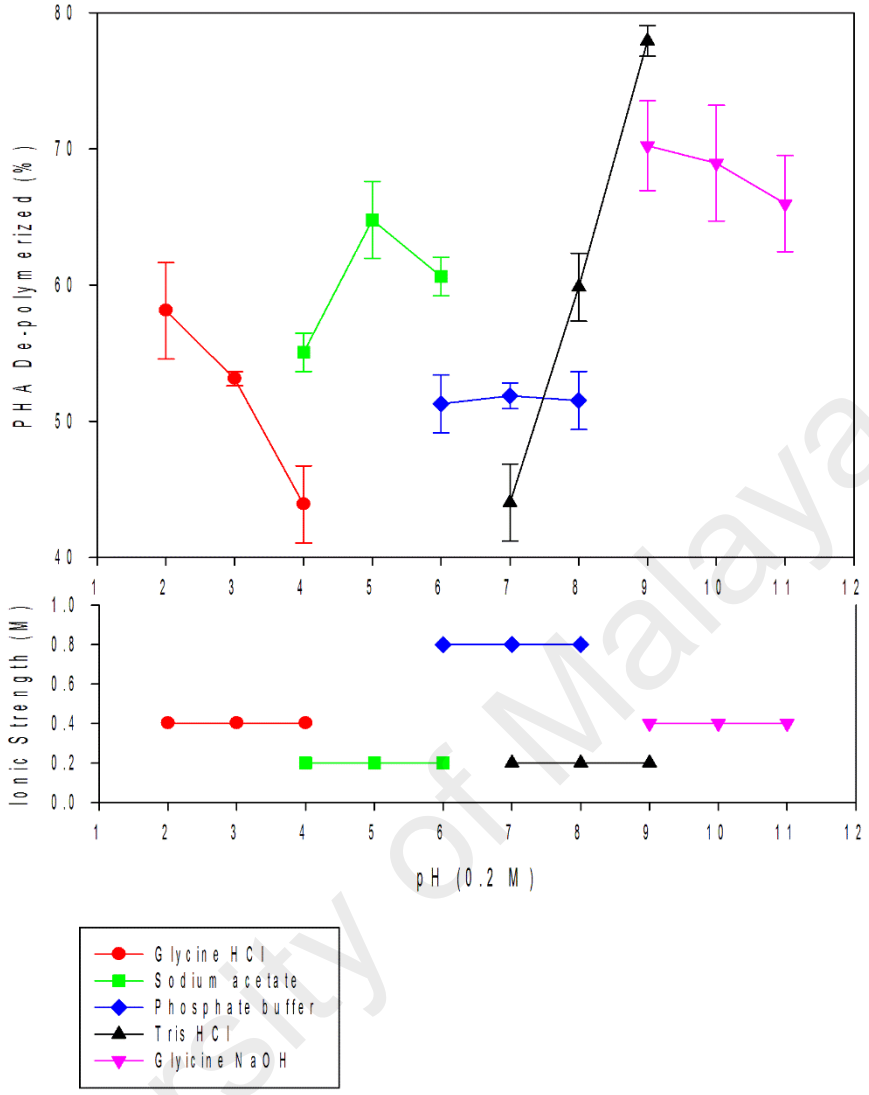


Figure 4.3a: Effects of different types of buffers on *in vivo* depolymerization of intracellular mcl-PHA. Values are means of three replications ± standard deviation. (Ionic strength, *I*: Glycine-HCl: 0.4 M; sodium acetate: 0.2 M; phosphate buffer: 0.8 M; Tris-HCl: 0.2 M; Glycine-NaOH: 0.4 M).

Table 4.2a: Effects of different types of buffers on *in vivo* depolymerization of intracellular mcl-PHA. (Ionic strength, *I*: Glycine-HCl: 0.4 M; sodium acetate: 0.2 M; phosphate buffer: 0.8 M; Tris- HCl: 0.2 M; Glycine-NaOH: 0.4 M).

Types of buffers	**pH**	**Initial PHA (g L^{-1})**	**Final PHA (g L^{-1})**	**PHA de-polymerized (%)**	**Total PHA de-polymerized (g L^{-1})**
Glycine-HCl	2	4.05 ± 0.10	1.70 ± 0.01	58.11 ± 3.50	2.35 ± 0.05
	3	3.61± 0.10	1.69 ± 0.01	53.12 ± 0.50	1.92 ± 0.05
	4	3.60 ± 0.20	2.02 ± 0.00	43.89 ± 2.80	1.58 ± 0.10
Sodium acetate	4	3.38 ± 0.05	1.53 ± 0.02	55.05 ± 1.41	1.87 ± 0.01
	5	3.46 ± 0.05	1.22 ± 0.01	64.79 ± 2.80	2.24 ± 0.00
	6	3.47 ± 0.10	1.37 ± 0.01	60.63 ± 1.40	2.10 ± 0.00
Phosphate buffer	6	3.71 ± 0.04	1.81 ± 0.02	51.28 ± 2.10	1.90 ± 0.01
	7	3.16 ± 0.00	1.52 ± 0.10	51.87 ± 0.92	1.64 ± 0.02
	8	3.04 ± 0.10	1.47 ± 0.08	51.53 ± 2.12	1.57 ± 0.10
Tris-HCl	7	3.73 ± 0.02	2.09 ± 0.06	44.03 ± 2.98	1.64 ± 0.01
	8	3.40 ± 0.02	1.37 ± 0.01	59.85 ± 2.55	2.04 ± 0.00
	9	3.44 ± 0.03	0.76 ± 0.00	77.93 ± 1.00	2.68 ± 0.01
Glycine NaOH	9	3.33 ± 0.02	0.99 ± 0.00	70.24 ± 3.32	2.34 ± 0.02
	10	3.43 ± 0.20	1.07 ± 0.01	68.95 ± 4.70	2.37 ± 0.02
	11	3.38 ± 0.05	1.15 ± 0.01	65.99 ± 3.85	2.23 ± 0.05

*Values are means of three replications ± standard deviation.

Different parameters such as temperature and buffer concentration were also investigated to improve the depolymerization performance in Tris-HCl. Figure 4.3b and Table 4.2b show the percentage of *in vivo* depolymerization of intracellular mcl-PHA at different temperatures from 25 to 40 °C. The highest activity of intracellular depolymerization was observed at 30 °C with 82.6 ± 0.7 wt% of *in vivo* mcl-PHA depolymerization after 24 hours incubation in 0.2 M Tris-HCl buffer.

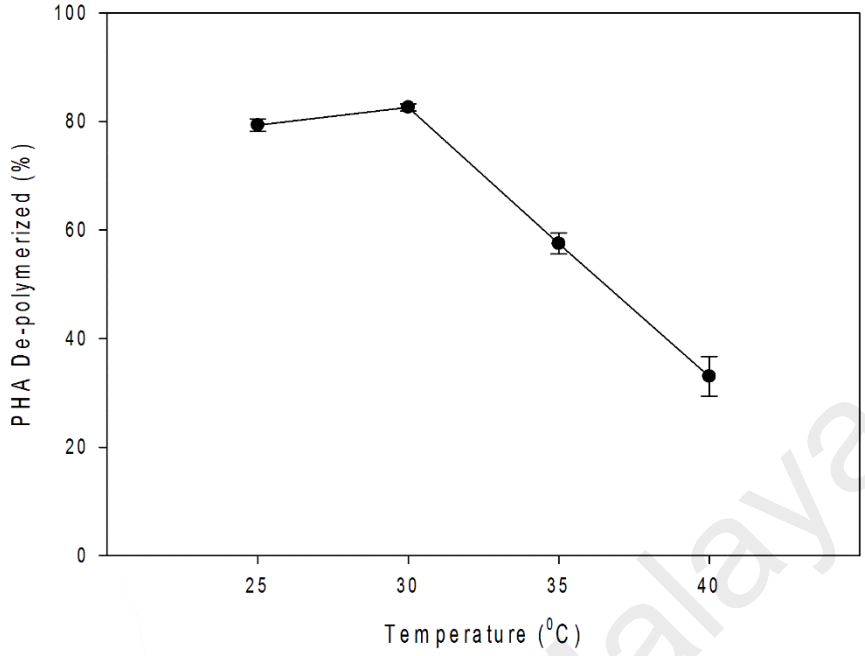


Figure 4.3b: Effects of different temperatures on *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and *I* = 0.2 M. Values are means of three replications ± standard.

Table 4.2b: Effects of different temperatures on *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9 and *I* = 0.2 M.

Temperature (ºC)	**Initial PHA (g L^{-1})**	**Final PHA (g L^{-1})**	**PHA de-polymerized (%)**	**Total PHA de-polymerized (g L^{-1})**
25	2.17 ± 0.05	0.45 ± 0.02	79.32 ± 1.11	1.72 ± 0.07
30	1.62 ± 0.11	0.28 ± 0.05	82.59 ± 0.69	1.34 ± 0.16
35	2.21 ± 0.60	0.94 ± 0.30	57.52 ± 1.93	1.27 ± 0.51
40	2.36 ± 0.70	1.58 ± 0.35	33.04 ± 3.66	0.78 ± 0.69

*Values are means of three replications ± standard deviation.

Figures 4.3c and Table 4.2c show the percentage of *in vivo* depolymerization of intracellular mcl-PHA at different molar concentrations of Tris-HCl buffer at 30 °C incubation temperature. From the results, *in vivo* depolymerization increased with higher concentration of Tris-HCl buffer from 0.025 until 0.2 M. *In vivo* depolymerization was observed to be in the range of 86.7 - 89.4 wt% with Tris-HCl buffer molarity from 0.2 to 0.5 M. To eliminate the possibility that *in vivo* depolymerization could be due to chemical hydrolysis reaction, control whole cells i.e. pre-treated bacterial culture with intracellular mcl-PHA (exposed to thermal treatment at 70 °C for 10 minutes before resuspended in Tris-HCl buffer with concentrations from 0.2 to 0.5 M) was examined, and *in vivo* depolymerization was found to be absent during the 24 hours incubation. The intracellular mcl-PHA content remained constant at 48.6 ± 1.9 wt% from the start till the end of the experiment. This was attributed to the thermal-related loss of depolymerization activities responsible for *in vivo* action towards the mcl-PHA.

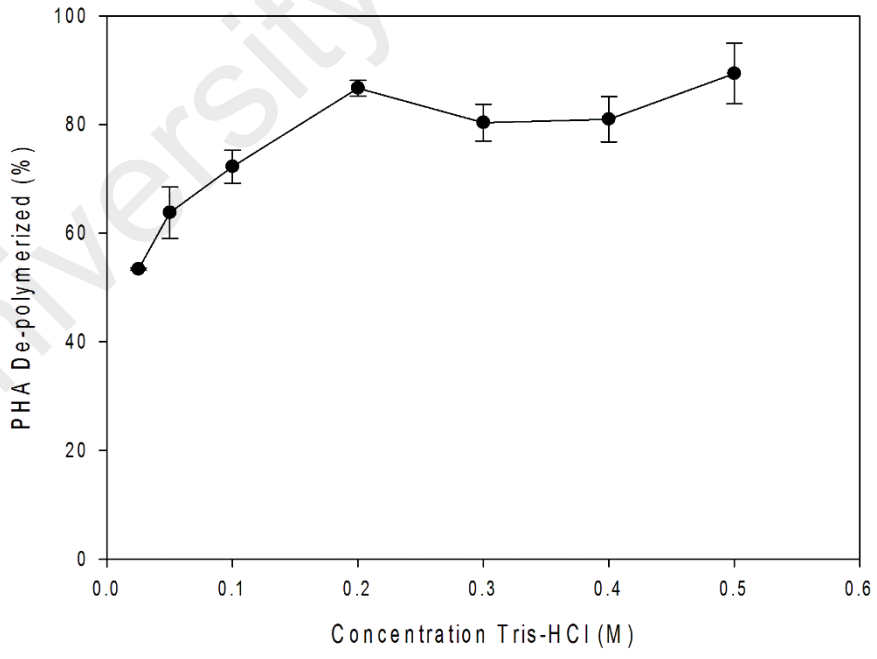


Figure 4.3c: Effects of different concentrations of Tris-HCl buffer, pH 9 at 30 ºC on *in vivo* depolymerization of intracellular mcl-PHA. Values are means of three replications ± standard deviation.

Table 4.2c: Effects of different concentrations of Tris-HCl buffer, pH 9 at 30 ºC on *in vivo* depolymerization of intracellular mcl-PHA.

Concentration Tris-HCl (M)	Initial PHA (g L^{-1})	Final PHA (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
0.025	3.20 ± 0.50	1.49 ± 0.00	53.36 ± 0.22	1.71 ± 0.20
0.050	3.33 ± 0.41	1.20 ± 0.01	63.77 ± 4.73	2.12 ± 0.22
0.100	3.24 ± 0.22	0.90 ± 0.11	72.25 ± 3.05	2.34 ± 0.51
0.200	3.62 ± 0.01	0.48 ± 0.01	86.70 ± 1.45	3.13 ± 0.02
0.300	3.64 ±0.03	0.72 ± 0.03	80.33 ± 3.42	2.92 ± 0.33
0.400	3.04 ± 0.40	0.58 ± 0.05	80.97 ± 4.16	2.46 ± 0.22
0.500	3.98 ± 0.60	0.42 ± 0.02	89.38 ± 5.61	3.56 ± 0.22

*Values are means of three replications ± standard deviation.

Time profile of *in vivo* depolymerization of intracellular mcl-PHA was determined in 0.2 M Tris HCl buffer, pH 9, I = 0.2 M incubated at 30 °C (Figure 4.3d and Table 4.2d). The *in vivo* depolymerization was shown to be near maximum after 42 hours incubation with 98.3 ± 0.7 wt% of mcl-PHA depolymerized. The initial pH was monitored until the completion of each incubation period and it did not show any significant changes (pH 9.0 ± 0.2) as was also reported by Ren et al. (2005), and attested to the strong buffering capacity of the buffer used. The DCW declined with the decreasing mcl-PHA content in total biomass after incubation for 42 hours.

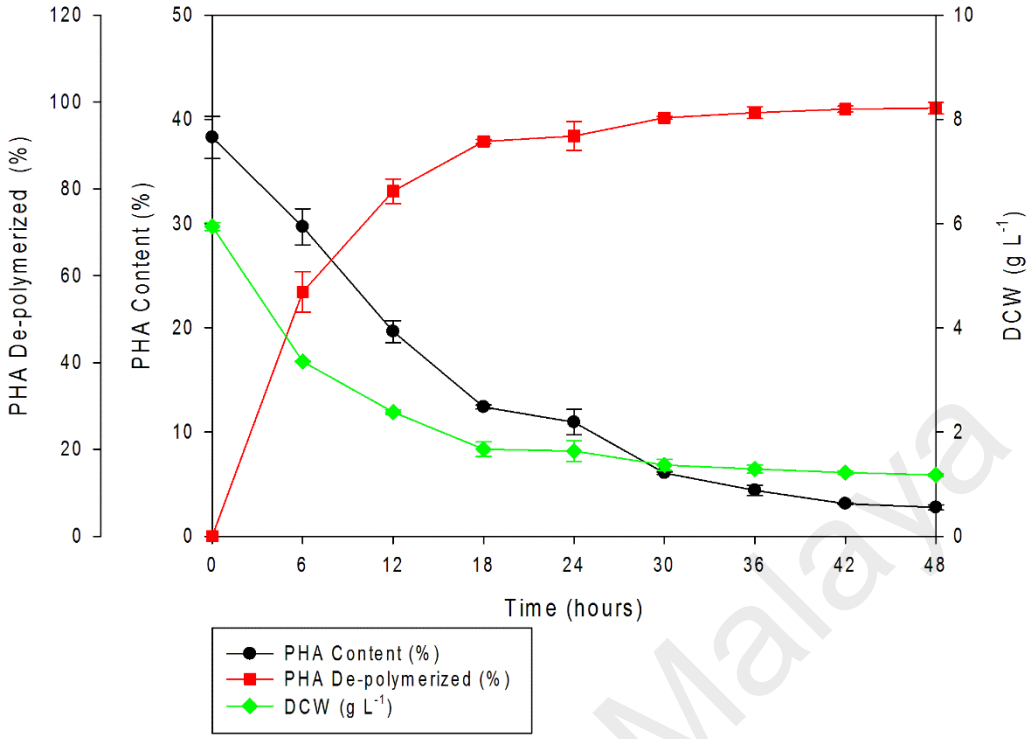


Figure 4.3d: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9, and I = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

Table 4.2d: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris-HCl buffer, pH 9, and I = 0.2 M at 30 °C.

Time (hour)	DCW (g L^{-1})	PHA content (wt%)	PHA content (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
0	5.94 ± 0.07	38.27 ± 2.02	2.27 ± 0.05	0	0
6	3.35 ± 0.00	29.68 ± 1.73	0.99 ± 0.10	56.26 ± 4.68	1.28 ± 0.11
12	2.38 ± 0.04	19.64 ± 1.16	0.47 ± 0.00	79.43 ± 2.86	1.81± 0.02
18	1.67 ± 0.14	12.40 ± 0.18	0.21 ± 0.02	90.90 ± 0.50	2.07 ± 0.05
24	1.63 ± 0.20	10.93 ± 1.22	0.18 ± 0.01	92.15 ± 3.30	2.10 ± 0.22
30	1.37 ± 0.11	6.07 ± 0.11	0.08 ± 0.01	96.36 ± 0.31	2.19 ± 0.01
36	1.29 ± 0.08	4.39 ± 0.50	0.06 ± 0.00	97.51 ± 1.36	2.22 ± 0.33
42	1.22 ± 0.03	3.11 ± 0.05	0.04 ± 0.00	98.33 ± 0.67	2.24 ± 0.22
48	1.17 ± 0.02	2.75 ± 0.25	0.03 ± 0.00	98.58 ± 1.32	2.24 ± 0.04

*Values are means of three replications ± standard deviation.

Figure 4.3e showed the amount of different monomers (µmol L^{-1}) liberated from *in vivo* depolymerization activities of mcl-PHA by bacterial culture suspended in 0.2 M Tris-HCl buffer, pH 9, I = 0.2 M at 30 °C. The cells initiated immediate depolymerization of intracellular mcl-PHA within the first six hours of incubation to release all types of available monomers and gradually continued to do so until it reached a plateau at 12 hours for C_6 and C_{12} monomers, and at 24 hours for C_8 and C_{10} monomers. Monomer liberation had ceased afterwards.

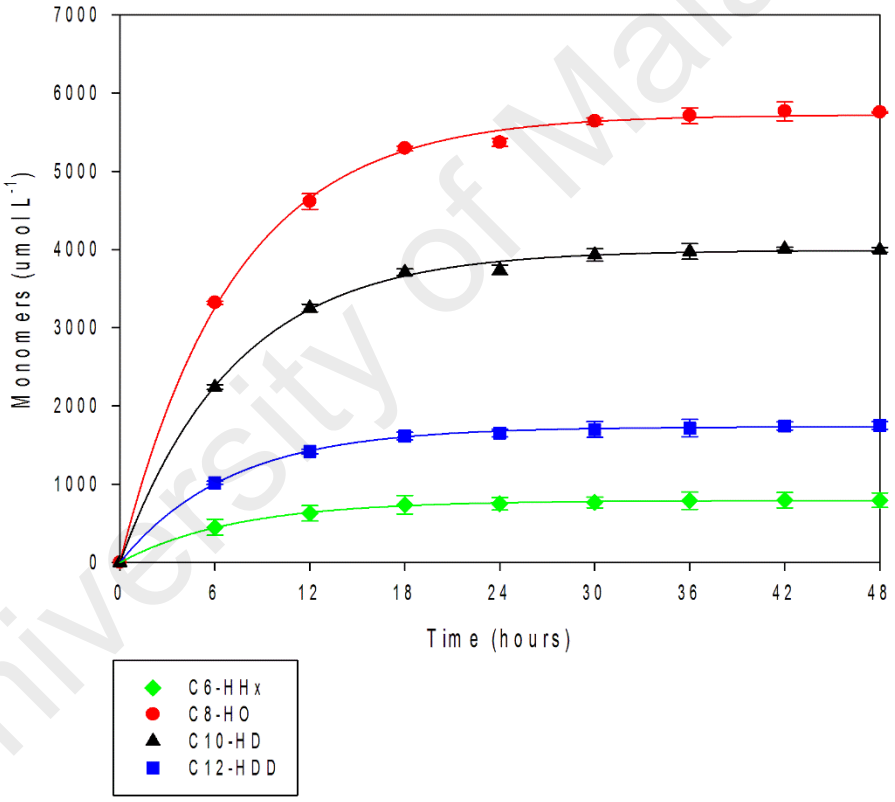


Figure 4.3e: Amount of different monomers liberated during *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9, and I = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

The total rate of mcl-PHA depolymerization was 0.21 g L^{-1} h^{-1} equivalent to 19.54 ± 0.09 U L^{-1} apparent depolymerization activity within 6 hours of incubation (Table 4.2e). The rate of depolymerization of different monomers ranged from 0.01 to 0.09 g L^{-1} h^{-1} corresponding to apparent depolymerization activities ranging from 1.26 to 9.22 U L^{-1}. Once again, the depolymerization activities were a direct reflection of the initial mol percentage of monomer distribution with the highest rate of depolymerization was observed for C_8 monomer followed by C_{10}, C_{12} and C_6 monomers (Table 4.2e).

Table 4.2e: Average volumetric rate of *in vivo* depolymerization of intracellular mcl-PHA and apparent enzymatic depolymerization activities towards different monomers in 0.2 M Tris-HCl buffer, pH 9, and *I* = 0.2 M at 30 °C during 6 hours incubation.

	Mcl-PHA	**C_6-HHx**	**C_8-HO**	**C_{10}-HD**	**C_{12}-HDD**
Initial (g L^{-1})	2.27 ± 0.20	0.10 ± 0.00	0.93 ± 0.03	0.82 ± 0.02	0.41 ± 0.02
Final (g L^{-1})	1.00 ± 0.17	0.05 ± 0.00	0.40 ± 0.04	0.37 ± 0.00	0.18 ± 0.04
Volumetric rate of depolymerization (g L^{-1} h^{-1})	0.21 ± 0.02	0.01 ± 0.00	0.09 ± 0.00	0.08 ± 0.00	0.04 ± 0.00
Apparent depolymerization activity (U L^{-1})	19.54 ± 0.09	1.26 ± 0.01	9.22 ± 0.05	6.23 ± 0.02	2.83 ± 0.01

*Values are means of three replications ± standard deviation.

In both MSM 3 and 0.2 M Tris-HCl buffer, pH 9, *I* = 0.2 M at 30 °C systems, the superficial preference of depolymerization towards C_8 monomer relative to other monomers present (as indicated by its higher hydrolysis rate) is most likely due to the higher initial mol fraction of C_8 monomer relative to the rest of monomer types present. For example, the initial C_8 monomer mol fraction (40.1 ± 2.7 mol%) was significantly higher than the next closest monomer fraction i.e. C_{10} (32.8 ± 2.8 mol%) (n = 6) (p < 0.05). Hence, the chances of C_8 to be liberated the most from the PHA granules by intracellular depolymerization are relatively larger than the others despite the assumption

that the different monomers are randomly distributed within the polymer chain.

From the average volumetric rate of depolymerization and depolymerization activities studied, it was also clear that *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 was highly active in 0.2 M Tris HCl buffer, pH 9, I = 0.2 M at 30 °C compared to the mineral media tested i.e. MSM 1, MSM 2 and MSM 3. It is evident that a suitable buffer solution provides a superior medium for *in vivo* process in *P. putida* Bet001 as shown by its relatively higher volumetric rate and apparent depolymerization activities towards *n*PHA and for each respective monomers present. As discussed earlier, the reason behind the observed effect is yet to be fully ascertained. From the results obtained, desirable rate and depolymerization activities for efficient *in vivo* depolymerization by whole cells be can be easily accomplished by manipulating the buffer properties such as type, concentration, pH and I in addition to other factors such temperature. This also opens up the possibility of adopting the aforementioned system for chiral molecules production in cell-free biology process (Swartz, 2012).

Morphological changes of the *P. putida* Bet001 cells before and after *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9, I = 0.2 M at 30 °C were observed under FESEM. Figure 4.3f **(**i) and (ii) showed the bacterial cells morphology before *in vivo* mcl-PHA depolymerization as coccobacilli-shaped entities. Figure 4.3f **(**iii) and (iv) showed the cells exhibiting shrivelled morphology with dimension reduction after *in vivo* depolymerization following the decrease in the number and size of mcl-PHA granules inside the cells.

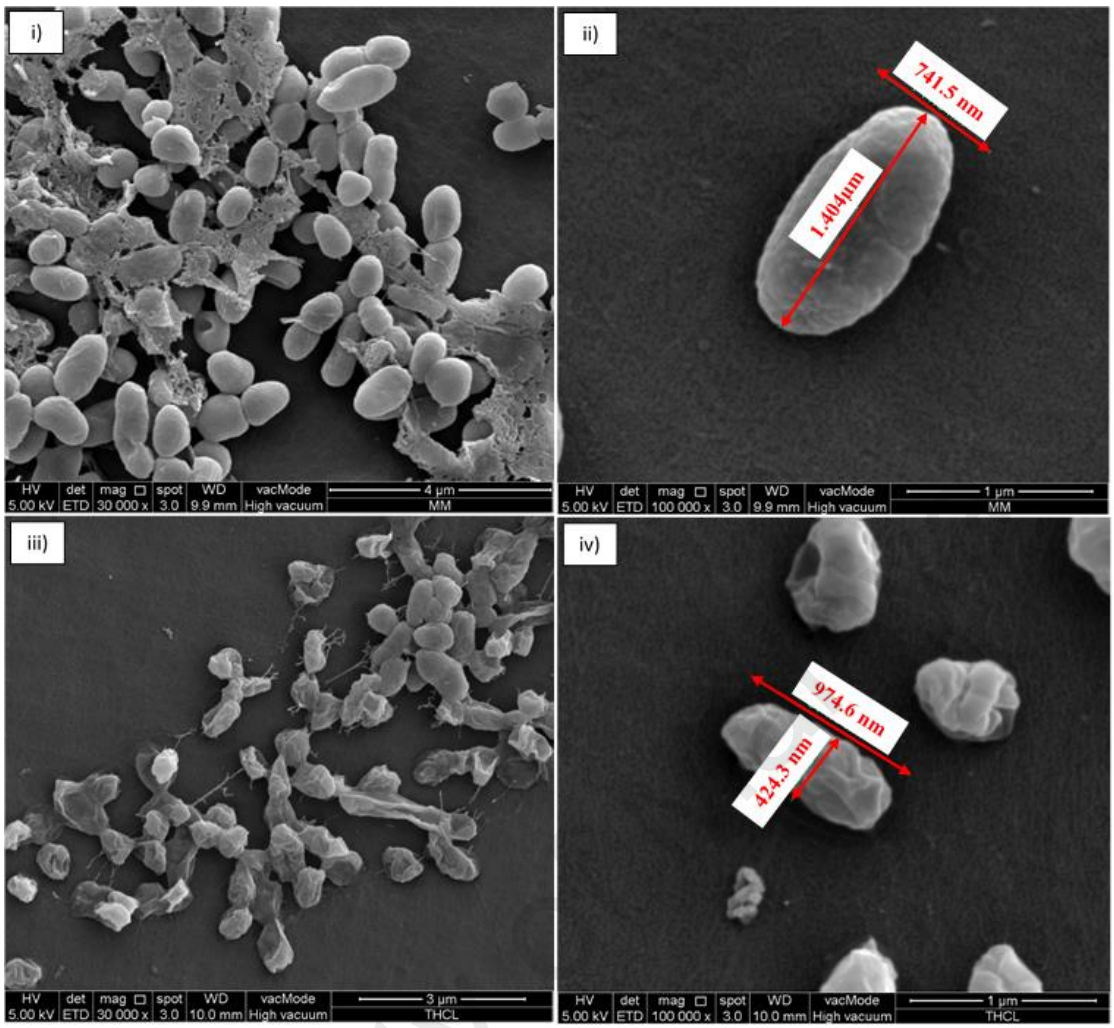


Figure 4.3f: Electron micrographs of *P. putida* Bet001 cells before [observed under (i) 30 000 & (ii) 100 000 × magnifications) and after [observed under (iii) 30 000 & (iv) 100 000 × magnifications) *in vivo* depolymerization of intracellular mcl-PHA.

4.2.3 Characterization

Characterization of remaining mcl-PHA following *in vivo* depolymerization using GPC, TGA, DSC and NMR was carried out to assess the properties of the polymer before and after depolymerization inside the bacterial cells. In addition, the data will assist to indicate the behavior of *in vivo* depolymerization of intracellular mcl-PHA.

GPC – the number average molecular weight (M_n) and weight average molecular weight (M_w) of the intracellular mcl-PHA before and after *in vivo* depolymerization as determined by GPC analysis is summarized in Table 4.3. The results shows that the molecular weights (M_n and M_w) of mcl-PHA decreased by 1.3- to 1.5-fold after *in vivo*

depolymerization. In addition, the polydispersity (M_w/M_n) index was shown to be almost similar for the cases studied (Table 4.3). Thus, it is hypothesized that the *in vivo* depolymerization follows an exo-type reaction which hydrolyzes ester bond of constituent monomers starting from terminal-end of polymer chain on the granule surface (Kawaguchi & Doi, 1992; Yoon & Choi, 1999). It also implies that the *in vivo* depolymerization is primarily confined to the surface of the *n*PHA granules and continues without significant perturbation to the whole granular structure, and leaving high molecular weight fragments inside the granule subsequent to the initiation of depolymerization process (Yoon & Choi, 1999). Nearly complete *in vivo* depolymerization of intracellular mcl-PHA by *P. putida* Bet001 whole cells was observed in 0.2 M Tris HCl buffer, pH 9, I = 0.2 M at 30 °C, with liberation of single monomers that matched the composition of *n*PHA, thus most likely points to exo-type reaction mechanism (*further discussion in section 4.2.4*).

Table 4.3: Molecular weight and thermal properties of mcl-PHA before and after *in vivo* depolymerization of intracellular mcl-PHA.

Mcl-PHA samples	**Molecular weight**			**Thermal properties**		
	M_n [a] **(Da)**	M_w [b] **(Da)**	**PDI** [c]	T_m [d] **(°C)**	T_d [e] **(°C)**	ΔH_m [f] **(J g^{-1})**
Before degradation	74 813	118 525	1.6	52.3	266.33	6.48
After degradation						
MSM lacking carbon source with 1 g L^{-1} ammonium (MSM 3)	47 306	91 328	1.9	50.0	269.00	5.07
0.2 M Tris-HCl, pH 9, 30 °C	53 305	82 435	1.6	47.7	278.32	4.07

[a]: Number average molecular weight
[b]: Weight average molecular weight
[c]: Polydispersity index
[d]: Melting temperature
[e]: Decomposition temperature
[f]: Heat of fusion

TGA – Table 4.3 and Figure 4.4a show the comparison of decomposition temperature (T_d) between the extracted mcl-PHA samples before and after *in vivo* depolymerization. Thermograms showed that all samples were thermally degraded in a single-step process between 250 – 300 ˚C. The polymer before *in vivo* depolymerization exhibited T_d at 266 ˚C, which was less thermally stable compared to after *in vivo* depolymerization. This suggested that extracted mcl-PHA samples from *in vivo* depolymerization consisted of a mixture of shorter chain length polymers that could assemble and packed closer to each other resulting in a more thermally stable configuration relative to the extracted original non-degraded mcl-PHA samples. The proposed effect was most obvious in samples of mcl-PHA obtained from *in vivo* depolymerization in 0.2 M Tris-HCl, pH 9, I = 0.2 M 30 °C with T_d at 278 ˚C, and less marked in samples after *in vivo* depolymerization in MSM 3 (T_d 269 ˚C).

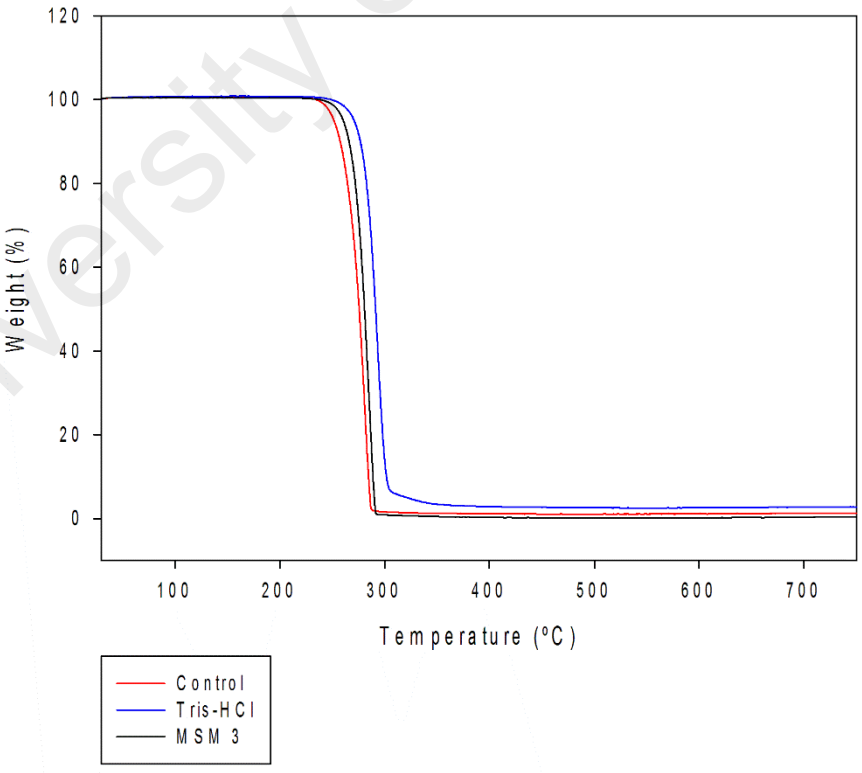


Figure 4.4a: TGA thermograms of extracted mcl-PHA samples before and after *in vivo* depolymerization of intracellular mcl-PHA.

DSC – the melting temperature (T_m) and enthalpy of fusion (ΔH_m) of the polymer samples before and after *in vivo* depolymerization were shown in Table 4.3. There was a slight reduction in melting temperature (T_m) and enthalpy of fusion (ΔH_m) of mcl-PHA after *in vivo* depolymerization indicating the altered nature of the extracted mcl-PHA samples after *in vivo* depolymerization.

^{1}H NMR – the spectra (Figure 4.4b) showed signals corresponding to the protons of the extracted mcl-PHA samples before and after (Tris-HCl, pH 9, I = 0.2 M 30 °C) *in vivo* depolymerization. The ^{1}H NMR spectrum after *in vivo* depolymerization was very similar to the one before *in vivo* depolymerization with signal peaks from both spectra displaying almost identical chemical shifts to the mcl-PHA spectra obtained from previous research on bacterial mcl-PHA production using lauric acid (Razaif - Mazinah et al., 2016). From the representative spectrum, the multiplet peaks at 2.5 ppm and triplet peaks at 5.2 ppm were assigned to methylene and methine protons of the α- and β-carbon respectively. The chemical shift at 0.9 and 1.2 ppm were assigned to the terminal methyl and methylene protons in the side chain of the polymers respectively. Chemical shift at 1.6 ppm was attributed to methylene protons adjacent to the β-carbon in the side chains of 3-hydroxyalkaonates (3HA) copolymer (Sin et al., 2010; Sin et al., 2011a; Gumel et al., 2012; Razaif - Mazinah et al., 2016). The signal intensity ratio of proton methine (5.2 ppm) group to proton methyl (0.9 ppm) group was reduced after *in vivo* depolymerization, which gives information on the occurrence of hydrolysis of ester linkages in mcl-PHA by intracellular depolymerization activities (Sin et al., 2010; Sin et al., 2011a).

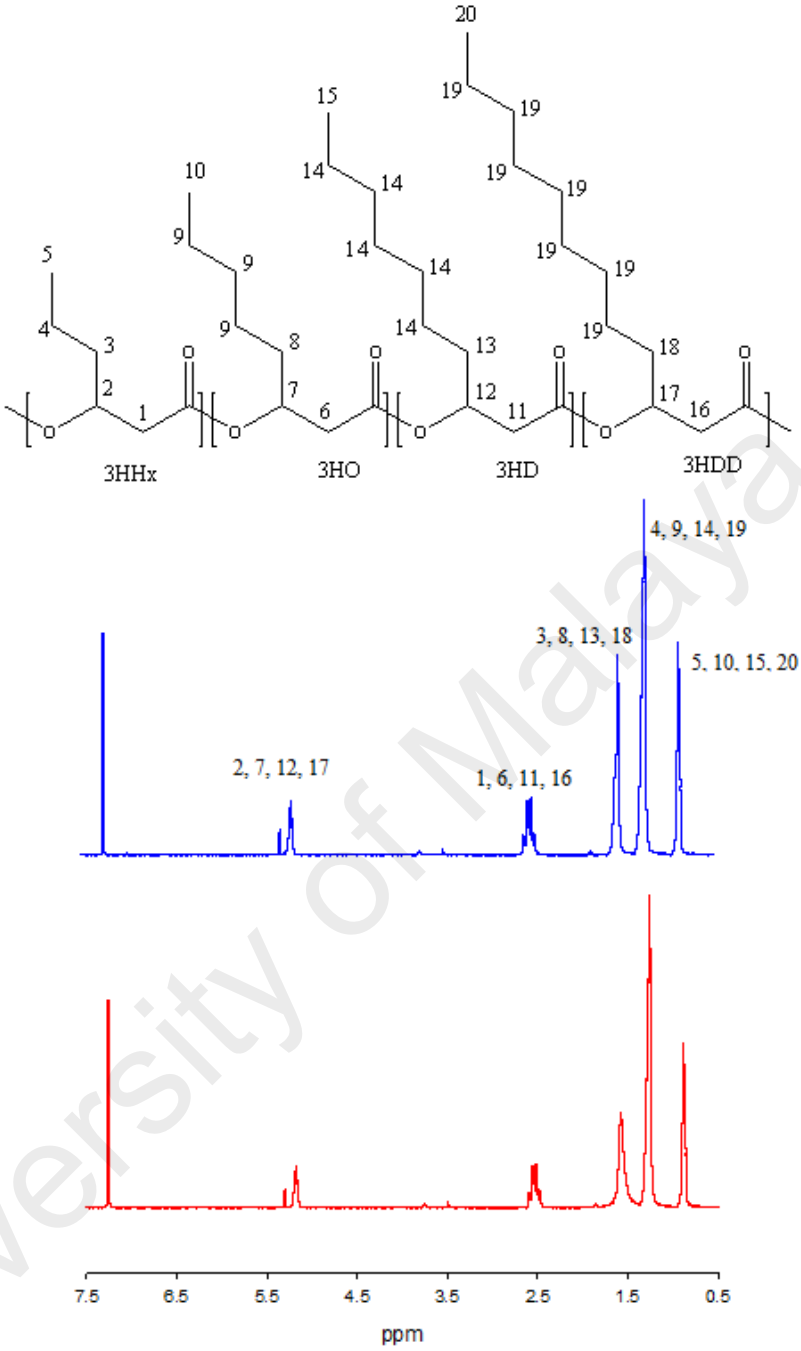


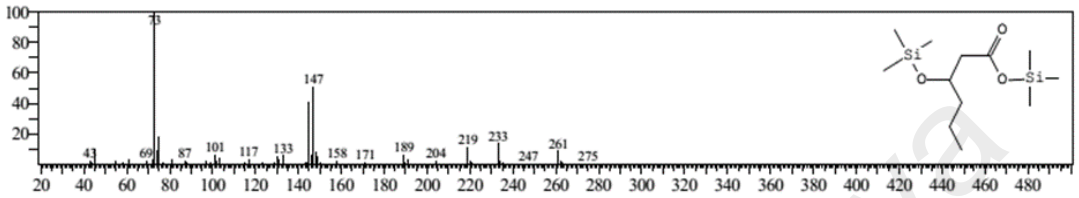
Figure 4.4b: Chemical structure of mcl-PHA, NMR (^{1}H) spectra of initial and after *in vivo* depolymerization of intracellular mcl-PHA.

4.2.4 Profiling of exogenous direct products from *in vivo* depolymerization of intracellular mcl-PHA

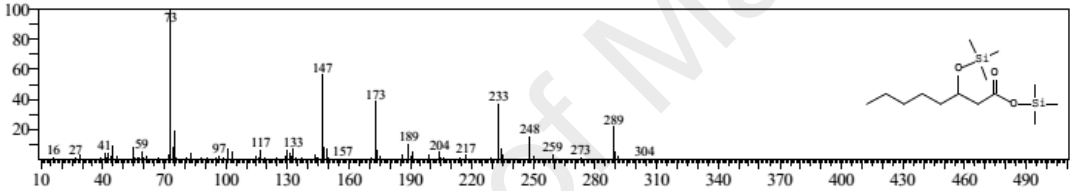
GCMS analysis also was carried out to detect the presence of exogenous direct products from *in vivo* depolymerization of intracellular mcl-PHA in the cell-free supernatant harvested from 0.2 M Tris-HCl, pH 9.0, I = 0.2 M, 30 °C buffer medium. Four major peaks corresponding to expected main products of *in vivo* depolymerization of intracellular mcl-PHA were successively obtained from ion fragments of mass spectrometry analysis and identified as 3-trimethylsiloxy esters of; hexanoic at *m/z* 261 (M-15), octanoic at *m/z* 289 (M-15), decanoic at *m/z* 317 (M-15) and dodecanoic at *m/z* 345 (M-15) acids. The ion impact mass spectra of 3-trimethylsiloxy esters are shown in Table 4.4a. The identities of the four single 3HAs detected in the cell-free supernatant matched all the 3HA monomers present in the intracellular mcl-PHA studied. It is likely that their presence in the supernatant originated from lysed cells and/or leaked out from the intact cells. It is also important to note that the presence of *in vivo* depolymerization products in the form of 3HA oligomers was not detected. This was observed for both silyllated- and non-silyllated samples from cell-free supernatant (*please refer appendix*). Hence, it was unlikely that silyllation of the extracted samples resulted in the degradation of possible oligomers to monomers. The detection of single 3HA monomers that matched the composition of *n*PHA in the cell-free supernatant has important implication in that it supported the hypothesis of exo-type reaction for intracellular mcl-PHA hydrolysis by depolymerization activities as discussed earlier. Tandem, successive hydrolysis of ester linkages by *in vivo* depolymerization starting from the granules surfaces generated single 3HA monomers corresponding to the composition of *n*PHA being degraded. The representative chemical structures of native mcl-PHA before depolymerization and individual 3HA monomers after *in vivo* depolymerization were shown in Figure 4.5.

Table 4.4a: Products of *in vivo* depolymerization of intracellular mcl-PHA were identified as a) Hexanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester; b) Octanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester; c) Decanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester; and d) Dodecanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester *via* NIST11 library, GCMS.

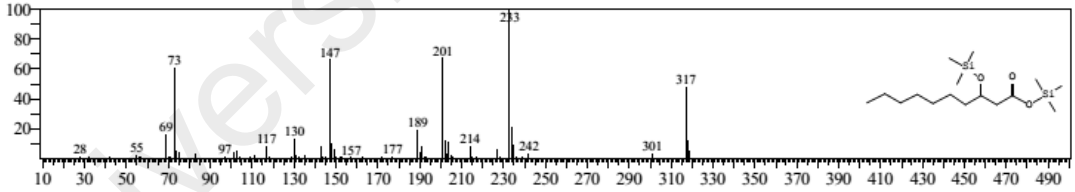
a) Hexanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
Formula: $C_{12}H_{28}O_3Si_2$
Molecular weight: 276



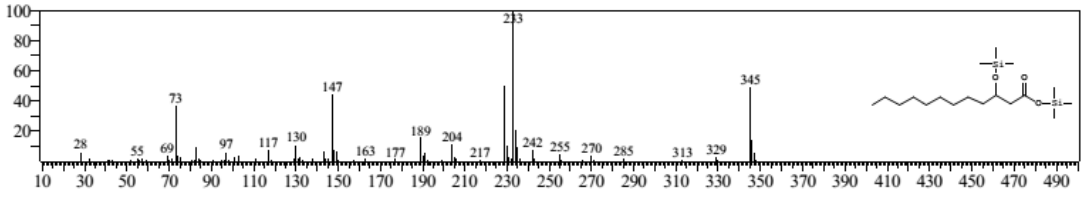
b) Octanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
Formula: $C_{14}H_{32}O_3Si_2$
Molecular weight: 304



c) Decanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
Formula: $C_{16}H_{36}O_3Si_2$
Molecular weight: 332



d) Dodecanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester
Formula: $C_{18}H_{40}O_3Si_2$
Molecular weight: 360



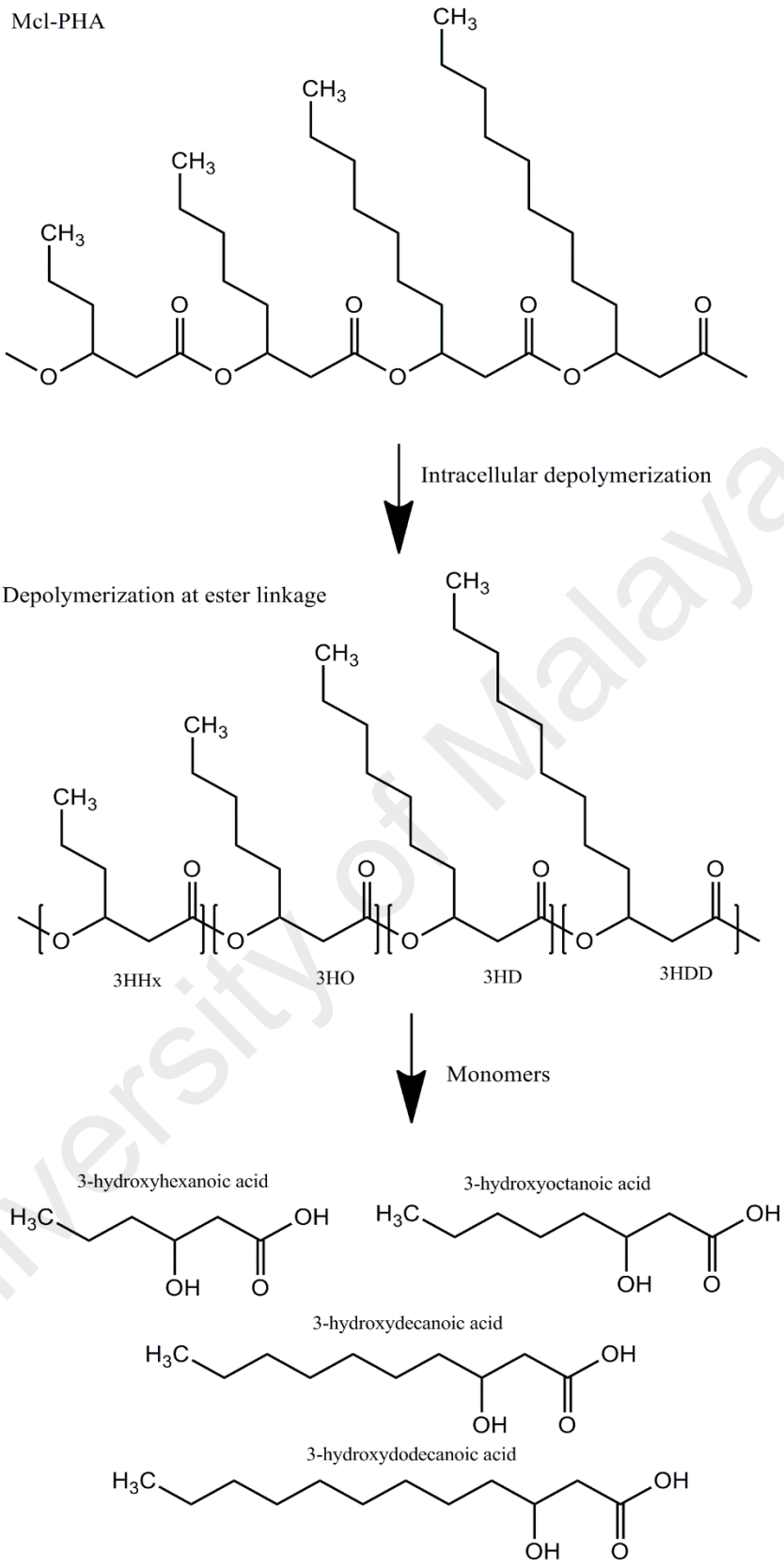


Figure 4.5: Chemical structures of native mcl-PHA before depolymerization and individual 3HA monomers structure after *in vivo* depolymerization.

It has been previously reported that (*R*)-3HAs with 8 to 18 carbon atoms length from mcl-PHA are more bactericidal than scl (*R*)-3HAs (Allen et al., 2012). Furthermore, (*R*)-3HAs are potential starting biomaterials for synthesis of antibiotics, vitamins, flavors and pheromones (Ren et al., 2005; Tokiwa & Ugwu, 2007). The yield of each of the four 3HA monomers was also calculated based on the spectral data obtained from the GCMS analysis (Table 4.4b). Interestingly, while the highest rate of depolymerization was calculated for 3HO, the results showed that 3HD monomer fraction to be almost completely liberated from *n*PHA (Table 4.4b). This was despite the fact that 3HD monomer fraction was quite comparable to the 3HO at 32.78 ± 2.83 mol% and 40.06 ± 2.74 mol%, respectively. Furthermore, in cases where a less complete liberation of monomers into the buffer medium was observed (3HHx, 3HO and 3HDD), it is likely that the residual monomers were channeled towards cell metabolism or have yet to be hydrolyzed from the granules. The reason for the particular observation made with regards to the 3HD fraction is yet to be clarified. It also highlights the gap in our understanding concerning the *in vivo* process. Elucidation would allow us to discover potential means to manipulate the depolymerization process itself.

Table 4.4b: Yields of exogenous direct products of mcl-PHA depolymerization.

Monomers ($\times 10^{-6}$ M)	**C_6-HHx**	**C_8-HO**	**C_{10}-HD**	**C_{12}-HDD**
Expected Yield	797	5753	3996	1752
Actual Yield	433	3965	3901	827
% Yield	54	69	98	47

*Standard deviation of measurement ± ≤ 13%.

4.3 *In vivo* and *In vitro* Depolymerizations of Intracellular Medium-Chain-Length Poly-3-hydroxyalkanoates

4.3.1 Preparation of crude reaction sample for *in vitro* depolymerization of intracellular mcl-PHA

Biosynthesis of mcl-PHA by *P. putida* Bet001 was carried out using lauric acid as sole carbon source. The mcl-PHA comprised of 3HHx (C_6) at 6.1 ± 2.0 mol%, 3HO (C_8) at 40.1 ± 2.7 mol%, 3HD (C_{10}) at 32.8 ± 2.8 mol%, and 3HDD (C_{12}) at 21.0 ± 3.2 mol%. The bacterial cells containing mcl-PHA was harvested after 48 to 72 hours fermentation by centrifugation. The cell suspension was subjected to lysis in ultrasonic bath under mild disruption conditions at frequency 37 kHz, 30 % of power output (Ishak et al., 2016), with temperature controlled < 25 ºC. The condition was adopted from previous research by Ishak et al. (2016) due to its apparent negligible effects towards the mcl-PHA main properties such as molecular weight. Effects of different sonication times on cell suspension were studied to determine the minimum time of insonation for complete cell lysis. Protein concentration was assayed using Bradford method to determine its liberation profile during cell lysis by ultrasonication. Figure 4.6a shows that liberation of cell protein content reached its maximum at 0.61 mg ml^{-1} concentration following 120 minutes of ultrasonication. The cell shape was observed before (Figure 4.6b.i-iii) and after lysis (Figure 4.6b.iv-vi) using confocal laser microscope under bright field and fluorescent imaging. Nile Red dye was used to stain intracellular mcl-PHA granules. The fluorescent dye showed high affinity towards intracellular lipid-like material such as PHA thus enabling direct observation to be made under fluorescence microscopy (Rumin et al., 2015). Both protein and microscopic analyses showed that the cells were completely lysed concomitantly releasing cellular content such protein after 120 minutes of ultrasonication. From micrographs taken before and after ultrasonication

(Figure 4.6b.i–vi), the general appearance of PHA granules released from lysed cells were observed to remain relatively intact.

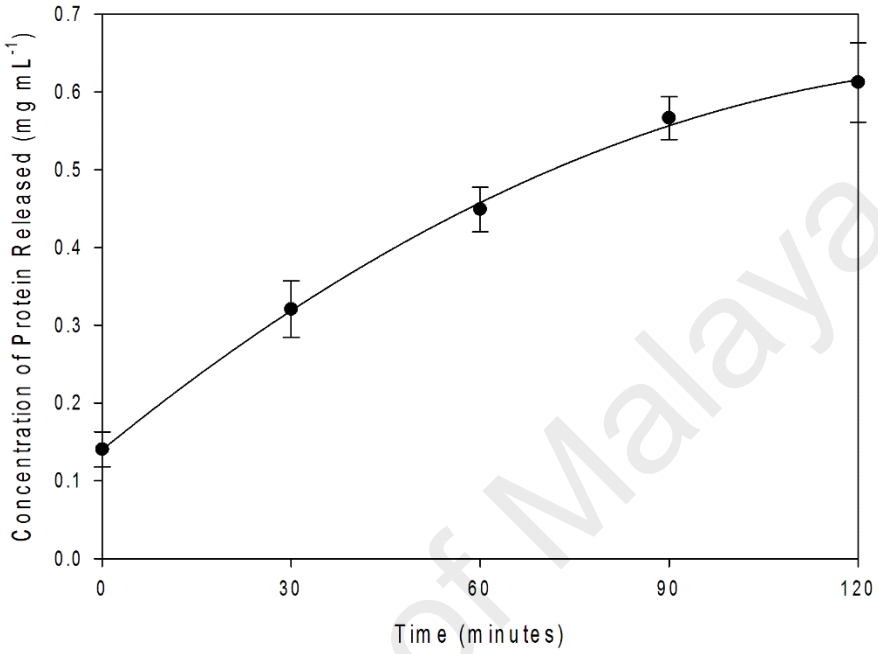


Figure 4.6a: Effects of sonication time on amount of protein released from lysed cells. Values are means of three replications ± standard deviation.

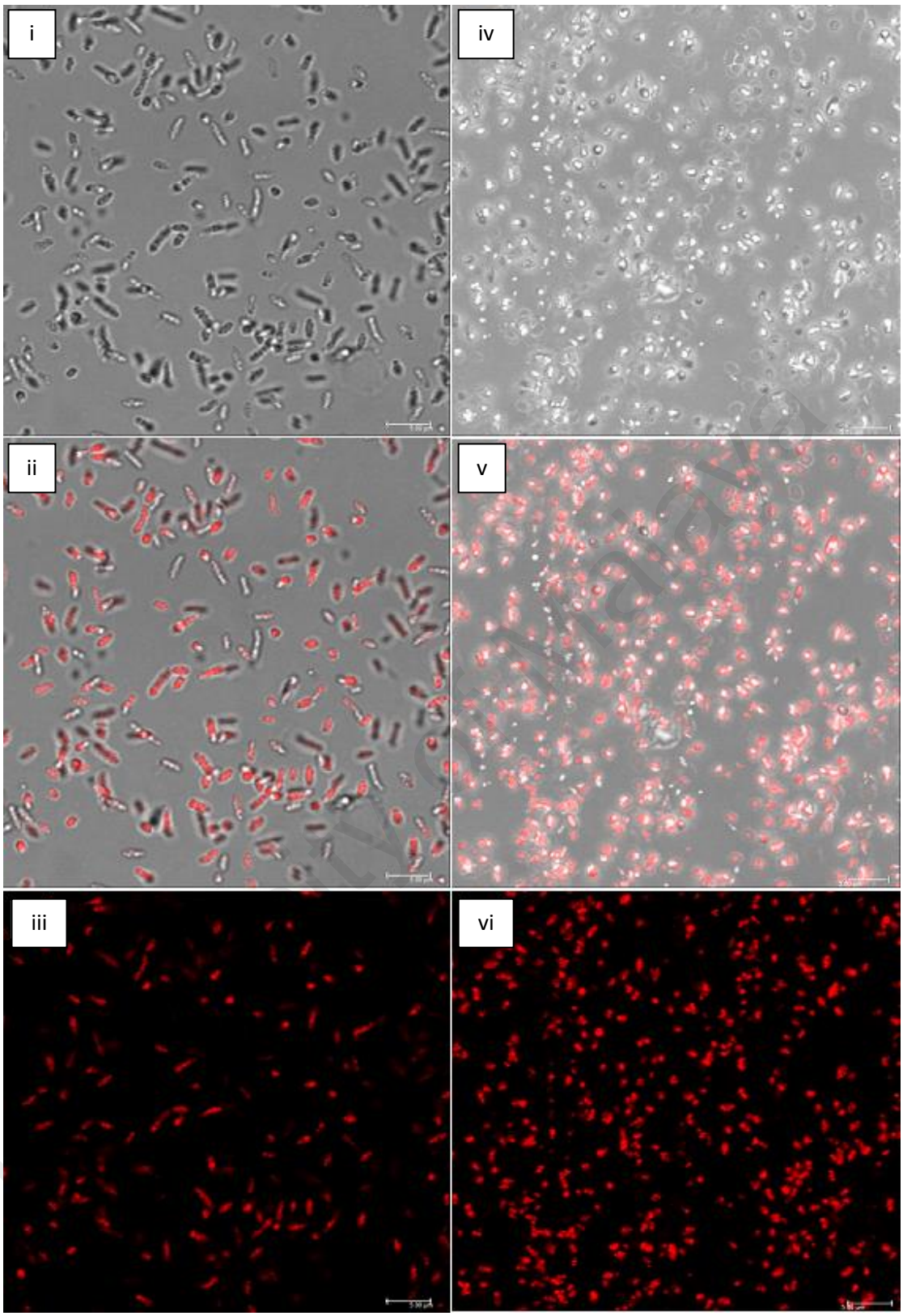


Figure 4.6b: Confocal laser micrographs of *P. putida* Bet001 cells before [observed under 3 × zoom 100 × magnification (i) brightfield image, (ii) overlay brightfield with confocal image, (iii) confocal image] and after [observed under 3 × zoom 100 × magnification (iv) brightfield image, (v) overlay brightfield with confocal image, (vi) confocal image] ultrasonication at 37 kHz, 30 % of power output, < 25 ºC, 120 minutes.

4.3.2 Effects of different sonication times on *in vitro* depolymerization of intracellular mcl-PHA

Different sonication times for the preparation of crude samples to be used in *in vitro* depolymerization studies were tested to investigate their effects on depolymerization activities. The crude sample preparations exposed to different sonication times (90, 120 and 150 minutes) at 37 kHz, 30 % of power output, < 25 ºC were collected and re-suspended in buffer solution 0.2 M Tris-HCl, pH 9 and I = 0.2 M at 30 °C (*obtained from earlier study*) for 48 hours. The results obtained in Figure 4.7 showed different sonication times had significant effect towards mcl-PHA depolymerization activities. The highest amount of *in vitro* depolymerization of intracellular mcl-PHA i.e. 51.3 ± 5.6 wt% [1.8 ± 0.1 g L^{-1} from initial PHA content 3.5 ± 0.0 g L^{-1} (Table 4.5)] was obtained from crude preparation of lysed cells following exposure to ultrasonication for 120 minutes. The lower mcl-PHA depolymerization in crude preparation obtained from cell suspension exposed to 90 minutes of sonication could be due to less efficient rupture of the cells. Exposure to ultrasound irradiation for 150 minutes could have denatured the depolymerization machinery of the mcl-PHA granules resulting in reduced depolymerization percentage (Figure 4.7). It is likely that exposure to ultrasound irradiation for 150 minutes or more may decrease the susceptibility of the granules to enzymatic hydrolysis. Hence, 120 minutes was selected as the appropriate sonication time for preparation of crude reaction sample exhibiting high mcl-PHA depolymerization activities. To eliminate the possibility that *in vitro* depolymerization could be due to the direct effect of ultrasound, control whole cells with intracellular mcl-PHA and suspended in Tris-HCl buffer were exposed to thermal treatment at 70 °C for 10 minutes prior to ultrasonication. The heat-treated cells were expected to exhibit insignificant *in vitro* depolymerization activities. Following ultrasonication at different times, the crude preparations of lysed cells were analyzed for depolymerization activities during 48-hours

incubation and they were found to be absent. The mcl-PHA content of the crude preparation remained constant at 37.4 ± 1.0 wt% throughout the experiment, and the percentage also represented an initial mcl-PHA content prior to ultrasound irradiation at different times. The absence of depolymerization activities was attributed to the thermal-related loss of enzymatic activities responsible for the *in vitro* action towards the mcl-PHA.

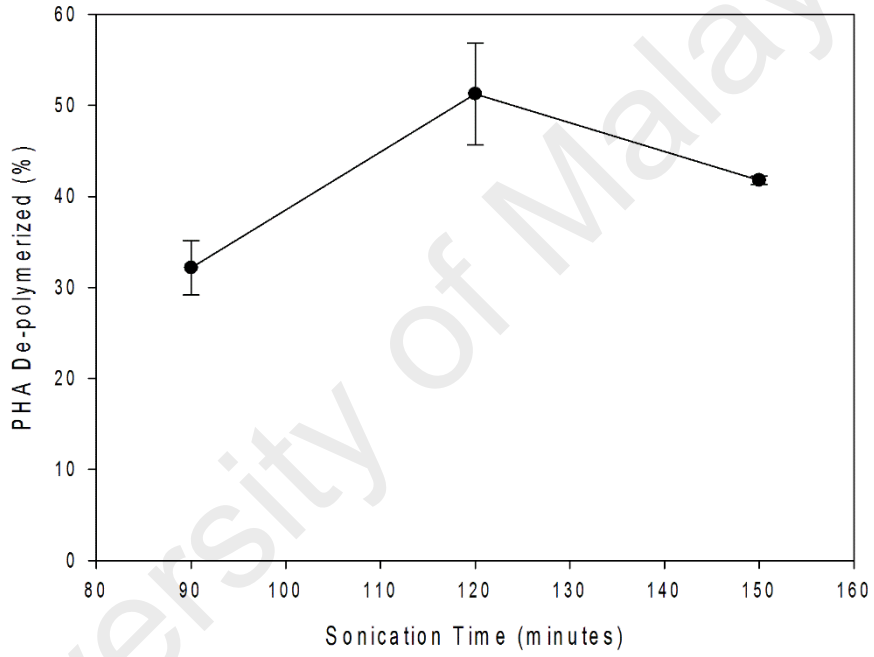


Figure 4.7: Effects of sonication time on *in vitro* depolymerization of intracellular mcl-PHA. Values are means of three replications ± standard deviation.

Table 4.5: Effects of sonication time on *in vitro* depolymerization of intracellular mcl-PHA.

Time (minutes)	Initial PHA (g L^{-1})	Final PHA (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
90	3.32 ± 0.05	2.26 ± 0.01	32.15 ± 2.90	1.06 ± 0.02
120	3.51 ± 0.02	1.72 ± 0.05	51.25 ± 5.62	1.79 ± 0.02
150	3.45 ± 0.01	2.01 ± 0.05	41.74 ± 0.40	1.44 ± 0.03

*Values are means of three replications ± standard deviation.

4.3.3 Comparison of *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA

A comparison between *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA was made. Bacterial cell culture after 48 to 72 hours of cultivation in the production medium were collected by centrifugation and re-suspended in buffer solutions of 0.2 M Tris-HCl, pH 9 and I = 0.2 M at 30 °C for *in vivo* depolymerization of intracellular mcl-PHA (*according to previous research*). On the other hand, crude preparation from ultrasonication of cell suspension were collected and re-suspended in identical buffer and conditions for *in vitro* depolymerization of intracellular mcl-PHA. The depolymerization profiles were shown in Figure 4.8a and b and Table 4.6a and b for *in vivo* and *in vitro* processes respectively. *In vitro* process showed lower rate and enzymatic depolymerization activities compared to the *in vivo.* The percentage of *in vivo* intracellular PHA depolymerization attained 92.2 ± 3.3 wt% whilst only 63.3 ± 1.0 wt% for *in vitro* process after 48 hours incubation. The average initial content of mcl-PHA in both preparations ranged from 33.6 ± 2.0 wt% (1.9 ± 0.1 g L^{-1}) to 38.3 ± 2.0 wt% (2.3 ± 0.6 g L^{-1}). The total biomass weight declined with the decreasing mcl-PHA content in the cells after incubation for 48 hours for *in vivo* process (5.9 ± 0.1 to 1.2 ± 0.0 g L^{-1}). For *in vitro* sample preparation, the initial cell concentration was measured at 7.7 ± 0.1 g L^{-1}, and 5.7 ± 0.2 g L^{-1} of cells were lysed (initial total pelleted fraction) following exposure to ultrasonication. After 48 hours of *in vitro* process, the gravimetric measurement of total pelleted fraction weight decreased to 1.8 ± 0.2 g L^{-1}.

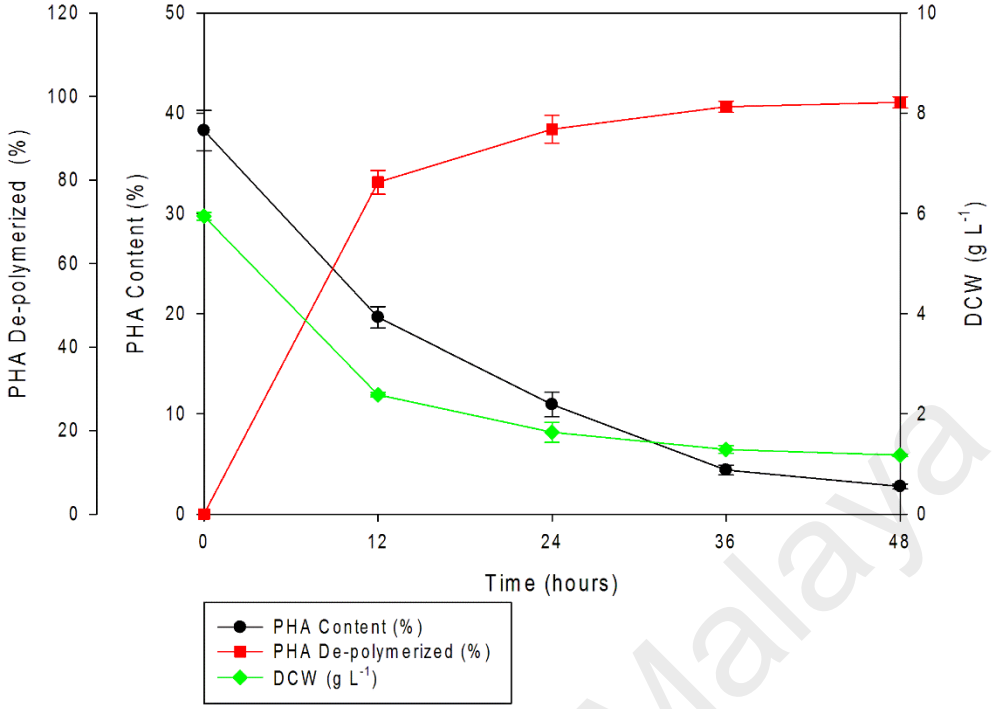


Figure 4.8a: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and I = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

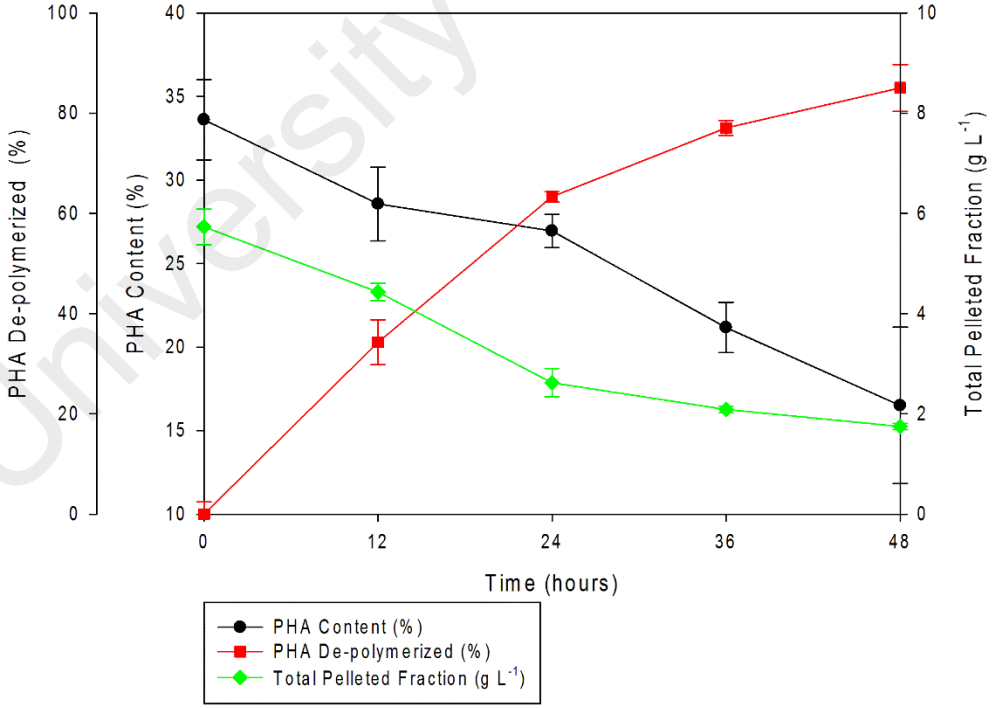


Figure 4.8b: Time profiles for *in vitro* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and I = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

Table 4.6a: Time profiles for *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and *I* = 0.2 M at 30 ºC.

Time (hour)	DCW (g L^{-1})	PHA content (wt%)	PHA content (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
0	5.94 ± 0.07	38.27 ± 2.02	2.27 ± 0.55	0.00 ± 0.00	0.00 ± 0.00
12	2.38 ± 0.04	19.64 ± 1.06	0.47 ± 0.05	79.43 ± 2.86	1.81 ± 0.02
24	1.63 ± 0.20	10.93 ± 1.22	0.18 ± 0.02	92.15 ± 3.30	2.10 ± 0.01
36	1.28 ± 0.07	4.39 ± 0.51	0.06 ±0.02	97.51 ± 1.36	2.22 ± 0.01
48	1.17 ± 0.02	2.75 ± 0.25	0.03 ± 0.01	98.57 ± 1.32	2.24 ± 0.02

*Values are means of three replications ± standard deviation.

Table 4.6b: Time profiles for *in vitro* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and *I* = 0.2 M at 30 ºC.

Time (hour)	DCW (g L^{-1})	PHA content (wt%)	PHA content (g L^{-1})	PHA de-polymerized (%)	Total PHA de-polymerized (g L^{-1})
0	5.73 ± 0.22	33.60 ± 2.00	1.93 ± 0.06	0.00 ± 2.40	0.00 ± 0.00
12	4.42 ± 0.32	28.56 ± 2.20	1.27 ± 0.08	34.29 ± 4.50	0.66 ± 0.02
24	2.61 ± 0.30	26.95 ± 1.00	0.71 ± 0.10	63.33 ± 1.02	1.22 ± 0.08
36	2.09 ± 0.08	21.17 ± 1.50	0.44 ± 0.10	77.04 ± 1.58	1.48 ± 0.01
48	1.75 ± 0.05	16.50 ± 0.45	0.29 ± 0.01	85.03 ± 4.60	1.64 ± 0.01

*Values are means of three replications ± standard deviation.

Figure 4.8c and d show the amount of different monomers liberated by depolymerization activities of intracellular mcl-PHA (µmol L^{-1}) from both *in vivo* and *in vitro* processes. The depolymerization activities were a direct reflection of the mol% of monomer distribution within the initial mcl-PHA with the highest rate of monomer liberation was observed for C_8 followed by C_{10}, C_{12}, and C_6. The direct products of depolymerization were identical for both *in vivo* and *in vitro* processes. For *in vivo* process, the cells initiated immediate depolymerization of intracellular mcl-PHA to liberate the available monomers and reached plateau at 12 hours for C_6 and C_{12}, and at 24 hours of incubation for C_8 and C_{10}, after which no further changes were observed i.e., mcl-PHA hydrolysis had ceased. In contrast, for *in vitro* process, the crude preparation of

mcl-PHA exhibited a more gradual depolymerization rate within 48 hours of incubation to release all types of available monomers.

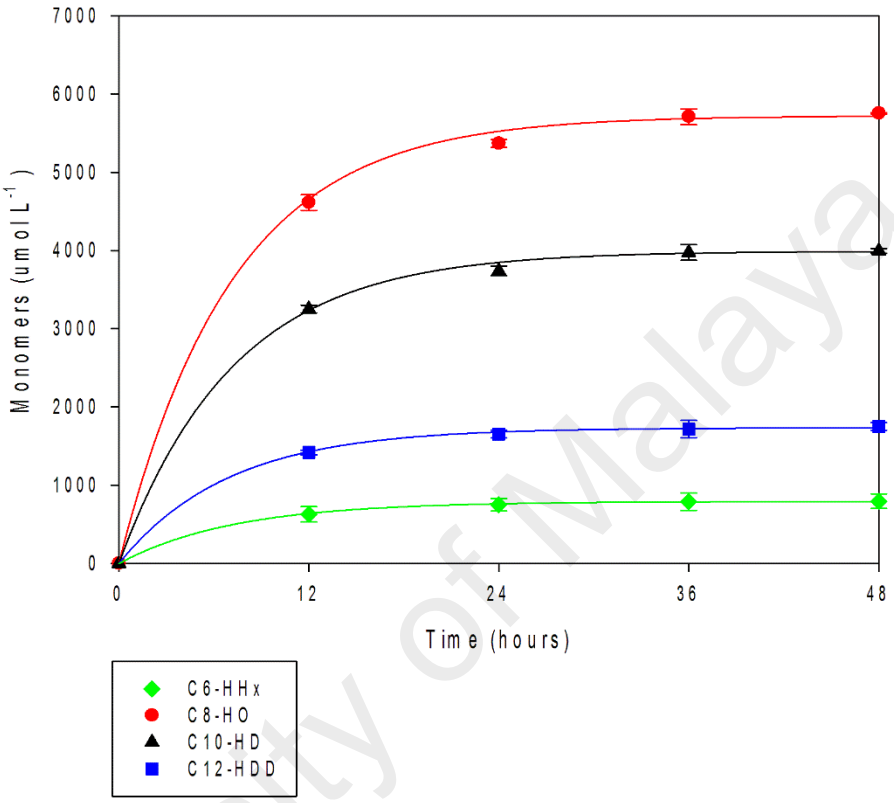


Figure 4.8c: Amount of different monomers liberated during *in vivo* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and I = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

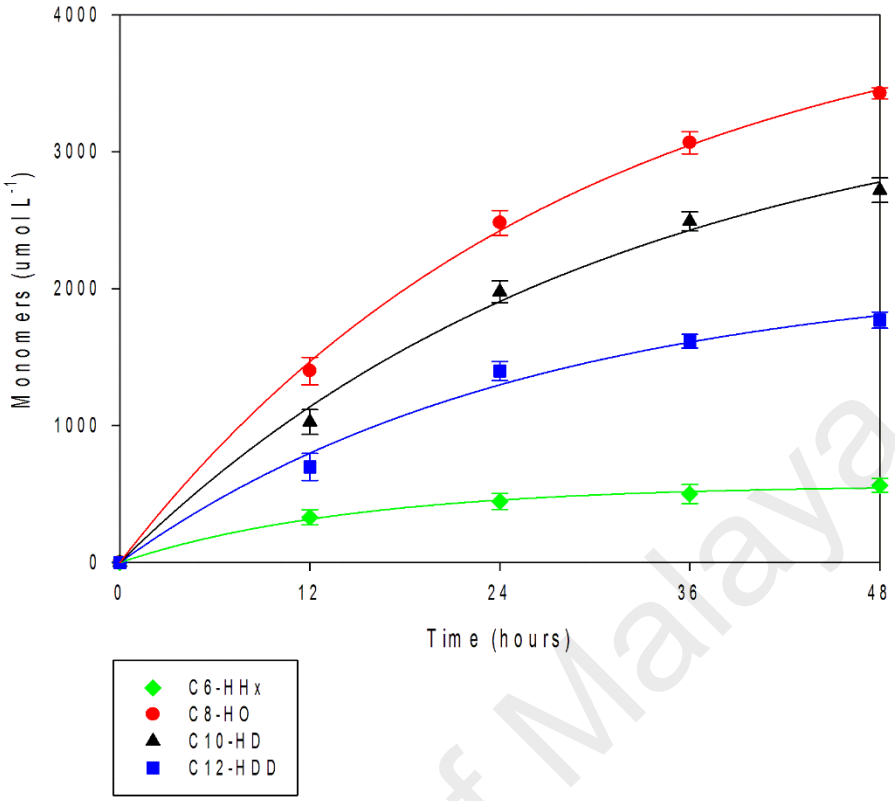


Figure 4.8d: Amount of different monomers liberated during *in vitro* depolymerization of intracellular mcl-PHA in 0.2 M Tris HCl buffer, pH 9 and *I* = 0.2 M at 30 ºC. Values are means of three replications ± standard deviation.

The comparison between average volumetric rates of *in vivo* and *in vitro* intracellular mcl-PHA depolymerization and apparent depolymerization enzyme activities towards different monomers in 0.2 M Tris-HCl, pH 9 and *I* = 0.2 M at 30 °C was made in Table 4.6c. The rate of *in vivo* mcl-PHA depolymerization was 0.15 ± 0.02 g L^{-1} h^{-1} equivalent to 11.98 ± 0.09 U L^{-1} apparent enzymatic depolymerization activity within 12 hours of incubation. In contrast, the rate of *in vitro* mcl-PHA depolymerization was 0.06 ± 0.02 g L^{-1} h^{-1} equivalent to 4.79 ± 0.09 U L^{-1} apparent enzymatic depolymerization activity within 12 hours of incubation. In addition, the rate of liberation of different monomers *in vivo* ranged from 0.01 to 0.06 g L^{-1} h^{-1} corresponding to apparent enzymatic depolymerization activities of 0.88 to 4.61 U L^{-1}. Conversely, lower rate of liberation of different monomers for *in vitro* process was determined i.e. ranging from

0.00 to 0.02 g L^{-1} h^{-1} corresponding to apparent enzymatic depolymerization activities of 0.46 to 1.94 U L^{-1}.

In other reported studies, *in vitro* depolymerization of intracellular *n*PHA had been investigated using granules isolated by glycerol density gradient centrifugation as well as centrifuged crude cell extracts without glycerol and incubating the obtained polymer fraction with hydrolytic enzymes methods; or making use of artificial PHB granules prepared according to the procedure described previously by Horowitz and Sanders (1994) and, Gebauer and Jendrossek (2006). Several factors have been studied to improve the *in vitro* depolymerization rate of *n*PHA e.g. by adding Mg^{2+} ions to increase ionic strength, pretreatment *n*PHA granule with trypsin or protease prior to subsequent hydrolysis by soluble *i*PHA depolymerase, or adding freshly soluble cell extract to *n*PHA (Foster et al., 1996; Chen et al., 2009). In contrast, the current study utilized cell-free biology of the crude preparation of ultrasonicated-lysed cells containing *n*PHA, *i*PHA depolymerase and other hydrolase enzymes, and more importantly the depolymerization of mcl-PHA to generate *R*3HAs was highly active in the *in vitro* system despite lower depolymerization rate compared to the *in vivo* whole cells process. The real advantage of the presented *in vitro* system as a potential route for chiral *R*3HAs production is the absence of multi-step pre-treatments on the crude reaction mixture obtained from mcl-PHA accumulating bacterial cell culture.

Table 4.6c: Average volumetric rate of *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA and apparent enzymatic depolymerization activities towards different monomers in 0.2 M Tris-HCl buffer, pH 9, and I = 0.2 M at 30 °C during 12 hours incubation.

	Mcl-PHA	**C_6-HHx**	**C_8-HO**	**C_{10}-HD**	**C_{12}-HDD**
***In vivo* Intracellular Depolymerization**					
Initial ($g\ L^{-1}$)	2.27 ± 0.20	0.10 ± 0.00	0.93 ± 0.03	0.82 ± 0.02	0.41 ± 0.02
Final ($g\ L^{-1}$)	0.47 ± 0.11	0.02 ± 0.00	0.20 ± 0.02	0.16 ± 0.03	0.09 ± 0.01
Volumetric rate of depolymerization ($g\ L^{-1}\ h^{-1}$)	0.15 ± 0.02	0.01 ± 0.00	0.06 ± 0.01	0.06 ± 0.01	0.03 ± 0.00
Apparent depolymerization activity ($U\ L^{-1}$)	11.98 ± 0.09	0.88 ± 0.01	4.61 ± 0.05	4.52 ± 0.02	1.97 ± 0.01
***In vitro* Intracellular Depolymerization**					
Initial ($g\ L^{-1}$)	1.93 ± 2.4	0.09 ± 0.00	0.71 ± 0.01	0.65 ± 0.01	0.48 ± 0.00
Final ($g\ L^{-1}$)	1.27 ± 4.7	0.05 ± 0.00	0.47 ± 0.00	0.44 ± 0.00	0.31 ± 0.00
Volumetric rate of depolymerization ($g\ L^{-1}\ h^{-1}$)	0.06 ± 0.02	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Apparent depolymerization activity ($U\ L^{-1}$)	4.79 ± 0.09	0.46 ± 0.01	1.94 ± 0.05	1.43 ± 0.02	0.97 ± 0.01

*Values are means of three replications ± standard deviation.

4.3.4 Characterization

GPC **–** Molecular weight data for *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA are shown in Table 4.7. Both processes exhibited decreasing molecular weight after depolymerization. The results showed that the average molecular weights (M_n and M_w) after *in vitro* process were lower than after *in vivo*. In addition, the polydispersity (M_w/M_n) index increased after *in vitro* depolymerization compared to initial samples and after *in vivo* depolymerization. The modest decrease in the molecular weight and the constant polydispersity of intracellular mcl-PHA during *in vivo* process could indicate an exo-type reaction (*as discussed earlier*) which hydrolyzes ester bond of constituent monomers starting from terminal-end of polymer chain on the granule surface resulting in high molecular weight fragments inside the granule subsequent to the initiation of depolymerization (Yoon & Choi, 1999). Conversely, for *in vitro* process the marked decrease in the average molecular weights and the higher polydispersity indicated that the liberated mcl-PHA samples from lysed cells were present as fragments with relatively wide molecular weight distribution. The possibility that ultrasound exposure may contribute to the fragmentation is not being ruled out at this stage. Nevertheless, depolymerization enzyme activities could still attack the fragments from their terminal end thus explaining the presence of single monomer types as the only detectable exogenous direct products from mcl-PHA depolymerization in the lysed cell, debris-free supernatant of *in vitro* process (*further discussion in section 4.3.5*).

Table 4.7: Molecular weight of mcl-PHA before and after *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA.

MCL-PHA samples	Molecular weight		
	M_n [a] (Da)	M_w [b] (Da)	PDI [c]
Before depolymerization	74 813	118 525	1.6
In vivo 24 hours	63 281	106 430	1.7
48 hours	49 101	84 760	1.7
In vitro 24 hours	41 469	109 450	2.6
48 hours	29 777	66 131	2.2

[a]: Number average molecular weight
[b]: Weight average molecular weight
[c]: Polydispersity index

TGA – Decomposition temperature for dried sample preparations containing mcl-PHA after *in vivo* and *in vitro* processes was determined from thermal analysis without the samples undergoing chloroform extraction. Control samples (thermally-treated whole cells with intracellular mcl-PHA; 70 °C for 10 minutes) and untreated cell samples without intracellular mcl-PHA were used as references. As can be seen in Figure 4.9a, both samples from *in vivo* and *in vitro* processes showed weight lost in the temperature range of 250 – 500 ˚C at decomposition temperatures of (T_d) 264.9 °C and 266.0 °C respectively. However, the T_d for untreated cell without mcl-PHA was lower at 246.3 °C whereas T_d for control samples was higher at 287.9 °C. It showed that the samples after *in vivo* and *in vitro* depolymerizations contained less polymer compared to control samples following depolymerization of intracellular mcl-PHA.

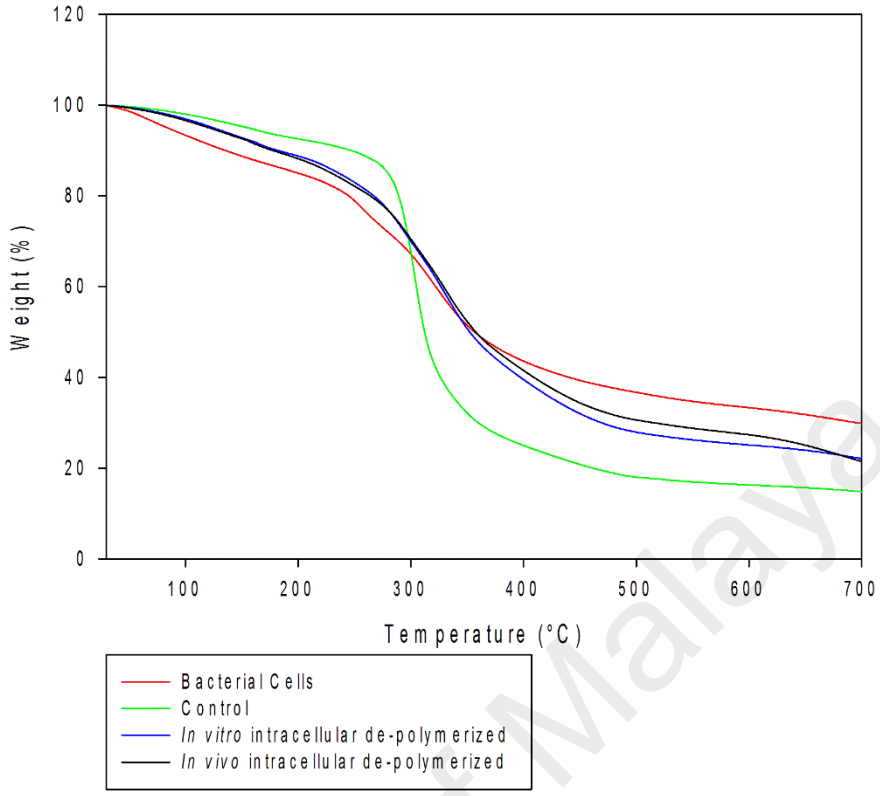


Figure 4.9a: TGA thermograms of dried sample preparations containing mcl-PHA after in *vivo* and *in vitro* depolymerizations of intracellular mcl-PHA.

^{1}H NMR **-** the spectra (Figure 4.9b) showed signals corresponding to the protons of the extracted mcl-PHA samples before depolymerization, and after *in vivo* and *in vitro* processes in Tris-HCl, pH 9 and I = 0.2 M at 30 °C. The ^{1}H NMR spectra obtained following both processes were very similar to the neat mcl-PHA spectra, where signal peaks from both spectra displaying almost identical chemical shifts to the spectra obtained from previous research on bacterial mcl-PHA production using lauric acid (Razaif - Mazinah et al., 2016). From the representative spectrum, the multiplet peaks at 2.5 ppm and triplet peaks at 5.2 ppm were assigned to methylene and methine protons of the α- and β-carbon respectively. The chemical shifts at 0.9 and 1.2 ppm were assigned to the terminal methyl and methylene protons in the side chain of the polymers respectively. Chemical shift at 1.6 ppm was attributed to methylene protons adjacent to the β-carbon in the side chains of 3-hydroxyalkaonates (3HA) copolymer (Sin et al., 2010; Sin et al.,

2011; Gumel et al., 2012; Razaif - Mazinah et al., 2016). The signal intensity ratio of proton methine (5.2 ppm) group to proton methyl (0.9 ppm) group was reduced after *in vivo* and *in vitro* depolymerizations, which supported the occurrence of ester linkages hydrolysis in mcl-PHA by enzymatic depolymerization activities (Sin et al., 2010; Sin et al., 2011).

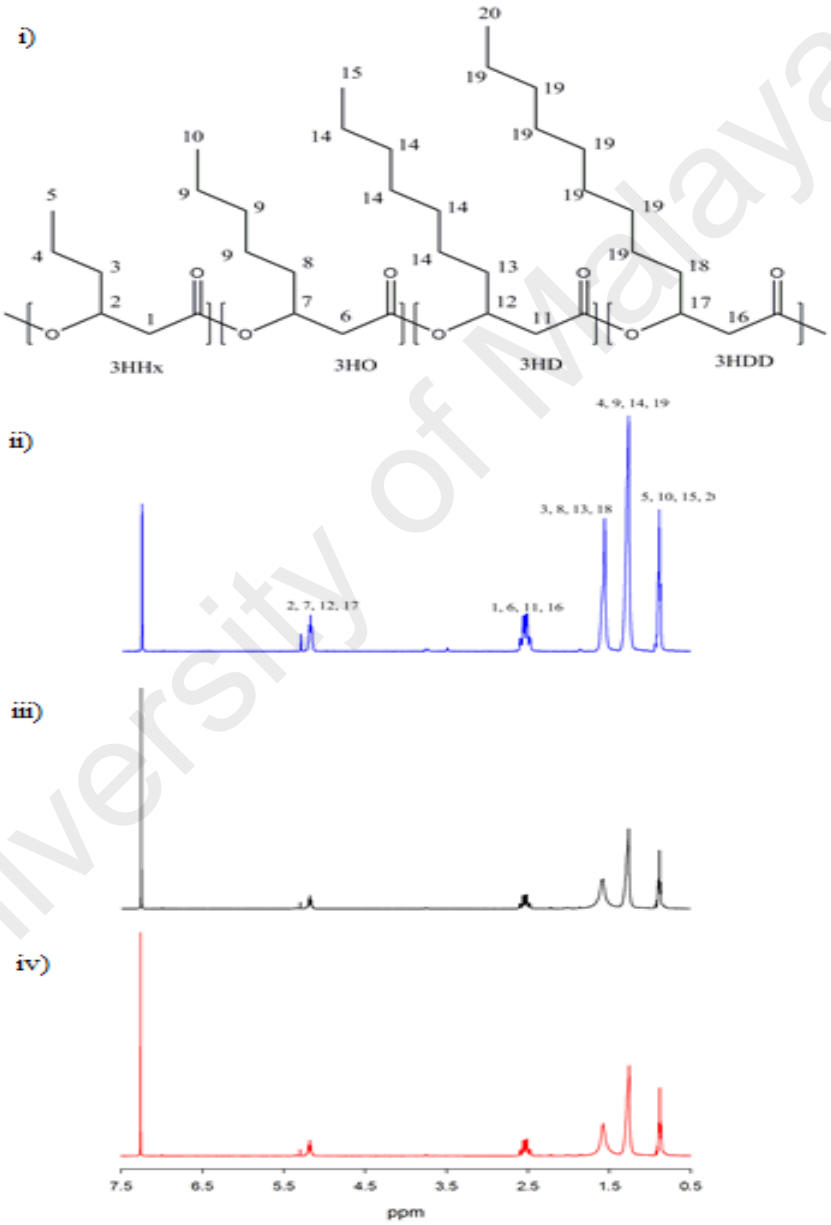


Figure 4.9b: Chemical structure of mcl-PHA (i) and NMR (^{1}H) spectra of initial mcl-PHA samples (ii), after *in vivo* (iii) and *in vitro* (iv) depolymerizations of intracellular mcl-PHA.

4.3.5 Profiling of exogenous direct products from *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA

The water-soluble compounds of exogenous direct products from both *in vivo* and *in vitro* depolymerizations were subjected to silylation for GCMS analysis. Silylation is the most established derivatization method as it readily volatizes the non-volatile sample by the addition of silyl groups to the free –OH groups. The silylated derivatives are more volatile and more stable for GC analysis (Orata, 2012). Four major peaks corresponding to main products of *in vivo* and *in vitro* processes were successively obtained from ion fragments of mass spectrometry analysis and identified as 3-trimethylsiloxy esters of; hexanoic at *m/z* 261 (M-15), octanoic at *m/z* 289 (M-15), decanoic at *m/z* 317 (M-15) and dodecanoic at *m/z* 345 (M-15) acids. The ion impact mass spectra of 3-trimethylsiloxy esters are shown in Table 4.4a (*identical to earlier study*). The fragmentation patterns for individual monomer were recorded and their retention times are shown in Table 4.8a.

Table 4.8a: GCMS fragmentation patterns for individual direct products from *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA.

Monomers	**Retention time (minute)**	**Mass-to-charge ratio (*m/z*)**	**Molecular weight**
Hexanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester ($C_{12}H_{28}O_3Si_2$)	7.265	73, 147, 233, 261, 275	276
Octanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester ($C_{14}H_{32}O_3Si_2$)	11.391	73, 147, 233, 289, 304	304
Decanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester ($C_{16}H_{36}O_3Si_2$)	15.536	73, 147, 233, 317	332
Dodecanoic acid, 3-trimethylsilyloxy, trimethylsilyl ester ($C_{18}H_{40}O_3Si_2$)	19.433	73, 147, 233, 345	360

All products were detected in the form of single type monomers only. The detection of single 3HA monomers that matched the composition of *n*PHA in the cell-free supernatant has important implication in that it supported the hypothesis of exo-type reaction for intracellular mcl-PHA hydrolysis by enzymatic depolymerization activities. Chen et al. (2009) also reported that almost all the *in vitro* hydrolytic products obtained from the depolymerization of intracellular amorphous PHA by *Bacillus megaterium* are 3HB monomers (Chen et al., 2009). The yield of each of the four 3HA monomers was also calculated based on the spectral data obtained from the GCMS analysis (Table 4.8b). Interestingly, while the rate of depolymerization was consistently higher for all types of monomers in *in vivo* process (Table 4.6c), significantly higher yield of monomer depolymerization was observed for *in vitro* process (Table 4.8b), with complete or near complete liberation of 3HD and 3HHx/3HO monomers, respectively. It is likely that in *in vivo* process, the competing metabolic pathway(s) may play important role in diverting away the yield since living whole cells were used. It also showed that whilst the *in vitro* process exhibited lower rate of depolymerization than the *in vivo*, the yields were comparatively better thus more attractive to be applied for the production of various types of mcl (*R*)-3HAs in a cell-free biology system.

Table 4.8b: Yields of exogenous direct products of mcl-PHA depolymerization.

Monomers ($\times 10^{-6}$ M)	**C_6-HHx**	**C_8-HO**	**C_{10}-HD**	**C_{12}-HDD**
***In vivo* Depolymerization**				
Expected Yield	797	5753	3996	1752
Actual Yield	433	3965	3901	827
% Yield	54	69	98	47
***In vitro* Depolymerization**				
Expected Yield	564	3426	2996	1771
Actual Yield	484	3242	3187	892
% Yield	86	95	~ 100	50

*Standard deviation of measurement ± 13%.

4.4 Process Components and Conditions for Biosynthesis, *In vivo* and *In vitro* Depolymerizations of Intracellular Medium-Chain-Length Poly-3-hydroxyalkanoates

From the results obtained, the working process components and conditions for biosynthesis, *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA by *P. putida* Bet001 were successfully determined. Table 4.9 shows the outcome of the determination:

Table 4.9: Working process components and condition for biosynthesis, *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA.

Components	**Conditions**		
	Biosynthesis	**Depolymerizations**	
		In vivo	***In vitro***
P. putida Bet001	From glycerol stock	From cell culture containing mcl-PHA	From cell culture containing mcl-PHA
Medium	1. Nutrient broth (NB) (100 ml flask, 30 ml medium), 2. Nutrient rich (NR) (250 ml flask, 100 ml medium), 3. Mineral medium (MSM) (250 ml flask, 100 ml medium) (Lauric acid C/N 20 as carbon and energy source)	0.2 M Tris-HCl buffer, pH 9, I = 0.2 M (250 ml flask, 100 ml medium)	0.2 M Tris-HCl buffer, pH 9, I = 0.2 M (250 ml flask, 100 ml medium)
Stages	Bacterial culture from glycerol stock was activated in NB, transferred to NR for cell growth, then transferred 1 g L^{-1} of cells to MSM for PHA accumulation	Cell culture harvested from fermentation, suspended in Tris-HCl buffer	Cell culture harvested from fermentation, lysed by ultrasonication (at 37 kHz, 30 % of power output, < 25 ºC, 120 minutes) suspended in Tris-HCl buffer
Incubation conditions	NB and NR for 24 hours, MSM for 48 hours (25 ˚C, 200 rpm agitation)	30 ˚C, 48 hours	30 ˚C, 48 hours

CHAPTER 5: CONCLUSIONS

P. putida Bet001 was used to study the biosynthesis of mcl-PHA. It was demonstrated that the bacterial strain showed good ability to produce mcl-PHA when supplied with different fatty acids. Highest mcl-PHA content and cell concentration were obtained under optimum cultivation conditions at C/N 20, 1 g L^{-1} initial cell concentration, 48 hours fermentation with lauric acid as sole carbon and energy source. Four different types of monomers were produced by the bacteria in lauric acid (C_{12}) *viz.* 3-hydroxyhexanoate (C_6), 3-hydroxyoctanote (C_8), 3-hydroxydecanoate (C_{10}) and 3-hydroxydodecanoate (C_{12}).

The bacterial cells containing mcl-PHA was used to study the *in vivo* depolymerization in different types of medium i.e. production medium and buffer solution. *In vivo* depolymerization showed dramatic effects on the PHA content, monomer composition and the chemical structure of intracellular mcl-PHA. *In vivo* depolymerization of intracellular MCL-PHA was found to be highly active in 0.2 M Tris-HCl buffer, pH 9, I = 0.2 M at 30 °C as compared to other buffers and production medium tested with monomer liberation rate reflected the mol% distribution of the initial polymer subunit composition. The study also suggested that parameters such as external pH and ionic condition play important role in the efficient depolymerization of intracellular mcl-PHA and subsequent liberation of hydrolyzed (*R*)-3HAs monomers. Sample characterizations before and after *in vivo* depolymerization alongside exogenous direct product analysis point to exo-type reaction for *in vivo* depolymerization of intracellular mcl-PHA. Individual monomeric products matched all the 3HA monomers present in the initial mcl-PHA before *in vivo* depolymerization in the bacterial cell.

For *in vitro* depolymerization of intracellular mcl-PHA studies, the optimum ultrasonication condition for preparation of cell-free biology system was found at 37 kHz frequency, 30 % of power output, < 25 ºC for 120 minutes. Cell-free biology system was

successfully applied in this research by using the crude lysed cell mixture from ultrasonication for *in vitro* depolymerization of intracellular mcl-PHA without further purification. The study also demonstrated a potential route towards a cost-effective method to produce chiral (*R*)-HA from *in vitro* depolymerization of intracellular PHA *via* cell-free biology system. The comparison between depolymerization activities in *in vivo* and *in vitro* setups was made. It was observed that *in vitro* depolymerization activities were lower compared to *in vivo*. *In vitro* depolymerization of intracellular mcl-PHA showed similar apparent monomer liberation rate compared to *in vivo* intracellular depolymerization of mcl-PHA. However, *in vitro* depolymerization showed higher final yields compared to *in vivo*. Sample characterizations before and after both *in vivo* and *in vitro* depolymerizations alongside exogenous direct product analyses point to exo-type reaction for both depolymerization processes.

The investigation provides important information on the fundamental characteristics of *in vivo* and *in vitro* depolymerization of intracellular mcl-PHA in *P. putida* Bet001. It is likely that similar physiognomies also exist in other fluorescent pseudomonad species. It is also shown that both *in vivo* and *in vitro* depolymerizations of intracellular mcl-PHA could be a potential biological route for the production of various types of mcl (*R*)-3HAs as platform chemicals. Mcl (*R*)-3HAs produced from the developed method can be further purified and applied as important building blocks in various fine chemical applications. Its primary advantage lies in the absence of complicated and often non environmental-friendly multistep upstream processes.

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PUBLICATIONS AND CONFERENCES

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